**Universidade do Minho**
Escola de Ciências da Saúde

Tatiana de Pinho Resende

# Temporal control of vertebrate embryo development: the role of Sonic Hedgehog in somite segmentation

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# Temporal control of vertebrate embryo development: the role of Sonic Hedgehog in somite segmentation

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# ABSTRACT / RESUMO

# ABSTRACT

All vertebrate species present a segmented articulated body, which is easily observed at the vertebral column level. This segmented nature can be detected quite early during embryonic development with the periodic formation of repeated segments, the somites, along the anterior-posterior embryo body axis. These are formed as blocks of cells that bud off from the rostral tip of the mesenchymal presomitic mesoderm (PSM), which flanks the embryo midline structures, notochord and neural tube. Somites will later originate all segmented structures of the adult body such as vertebrae, intervertebral disks, ribs, the dermis of the back and all skeletal muscles, except those of the head.

Somitogenesis occurs in a highly controlled and coordinated fashion and both the number of somites and the periodicity with which they are formed are constant and species specific. In the trunk region of the chick embryo a new pair of somites is formed every 90min. Underlying somite segmentation periodicity is an intrinsic molecular oscillator designated segmentation molecular clock. It was first described in the chick embryo with the demonstration of *hairy1* cyclic expression in the PSM with a periodicity of 90min, which corresponds to the time required to form a pair of somites in the chick. It is now known that several genes belonging to distinct signaling pathways such as Notch, Wnt and Fgf present a similar oscillatory behavior. Periodic gene transcription has been described to occur in other vertebrates, other tissues and also in cultured cells. This suggests that the molecular events underlying somitogenesis are highly conserved and that gene oscillations may be a widespread mechanism experienced by many cells and tissue types. A second molecular regulation has been described to account for period somite formation, the wavefront of differentiation. PSM maturation is defined by two opposing gradients with cross-regulatory activities: high Fgf/Wnt levels maintain the posterior PSM in an undetermined state and are counteracted by an anterior gradient of Retinoic Acid (RA). The confrontation between these opposing gradients and the molecular clock oscillations regulates somite formation in the anterior PSM. Thus, although the molecular clock operates along the entire PSM, only its anterior third is determined to form somites. Within this PSM region, only the medial-most PSM (M-PSM) possess intrinsic information for both somite formation and molecular clock gene expression, suggesting that M-PSM and lateral PSM (L-PSM) cells are differently committed to segment.

Both somite formation and somitogenesis molecular clock are thought to operate independently of the embryo midline structures, notochord and neural tube, and the signaling molecules produced therein, namely Sonic hedgehog (Shh). Shh is the most studied member of the Hedgehog family, which has been implicated in several mechanisms during embryo development

but has never been associated with somitogenesis regulation. However, quail/chick grafting experiments have suggested that the midline structures regulate symmetrical bilateral somite formation. Moreover, Shh knock-out mice lack the entire vertebrate column except for five to six ribs.

In the present study, we have investigated the role of midline derived Shh in somitogenesis regulation. We show that chick PSM cells express both Shh receptors *smoothened* and *patched*, enabling them to respond to notochord-derived Shh. Upon notochord ablation, we observe a delay in somite formation accompanied by an increased period of the molecular clock oscillations. These alterations are recapitulated by Shh chemical inhibitors and rescued by an exogenous Shh source, indicating that Shh is the notochord-derived signal responsible for those perturbations. Segmentation rate recovers over time, accompanied by *raldh2* overexpression. Accordingly, external RA supply rescues somite formation. Shh absence leads to an upregulation in the PSM of its downstream effectors, the Glis, in a repressor form and RA is thought to counteract their activity. We have also addressed the role of Shh in the differential specification of M- and L-PSM. We show that a diffusible signal travels along the M-L anterior PSM axis and that Shh pathway is responsible for the recruitment of lateral cells by medial ones for timely somite formation. Quail/chick grafts experiments indicate that prospective L-PSM can be re-specified into a medial fate when placed into a PM-PSM position and we suggest that this is also mediated by Shh. A model for Shh activity during PSM specification and somitogenesis, as well as interactions with the diverse pathways operating in the PSM is proposed. Altogether, the results presented here provide concluding evidence that Shh signaling is a component of the intricate molecular machinery responsible for temporal control of somite formation, implicating this molecule in the somitogenesis machinery for the first time.

# RESUMO

Os vertebrados são animais segmentados, o que é evidenciado cedo no desenvolvimento embrionário com o aparecimento de estruturas metaméricas, os sómitos, ao longo do eixo anterior-posterior do embrião. Estes formam-se periodicamente como blocos de células a partir da parte rostral da mesoderme pré-somítica (MPS), que surge como duas bandas de tecido mesenquimatoso a ladear as estruturas axiais do embrião, a notocorda e o tubo neural. Os sómitos originam todas as estruturas segmentadas presentes no animal adulto: vértebras, discos intervertebrais, costelas, a derme das costas e todos os músculos esqueléticos do tronco e membros.

A somitogénese é um processo coordenado e tanto o número total de sómitos como o tempo necessário para a formação de cada par é constante e específico de cada espécie. Na região do tronco da galinha, um novo par de sómitos é formado a cada 90min. A regular a surpreendente periodicidade da formação de sómitos está o relógio molecular da segmentação, que foi primeiramente descrito em galinha aquando da observação de que o gene *hairy1* apresenta um padrão de expressão cíclico. Este tem uma periodicidade de 90min, o que corresponde ao tempo necessário para se formar um novo par de sómitos na galinha. Actualmente sabe-se que diversos genes pertencentes a vias de sinalização como Notch, Wnt e Fgf apresentam também comportamento oscilatório. Esta transcrição periódica foi igualmente descrita noutros vertebrados, noutros tecidos e em linhas celulares, sugerindo que os mecanismos moleculares subjacentes à somitogénese são conservados e que este comportamento oscilatório pode ser um evento generalizado, ocorrendo em diferentes células e tecidos. A formação periódica de sómitos é também regulada por uma frente de maturação observada na MPS e definida por dois gradientes opostos: a MPS posterior é mantida num estado indiferenciado por elevados níveis de Fgf/Wnt, que são contrapostos por um gradiente anterior de ácido retinóico (AR). O confronto entre este gradiente de maturação e as oscilações do relógio regulam a formação de sómitos na MPS anterior. Deste modo, apesar de o relógio da somitogénese estar activo em toda a MPS, apenas a sua porção anterior está determinada para segmentar. Nesta região, verifou-se também que apenas a porção mais mediana da MPS (M-MPS) contém informação intrínseca para a formação de sómitos e a expressão de genes do relógio, o que sugere que a MPS-M e a MPS lateral (MPS-L) são diferentes no que diz respeito à sua capacidade de segmentação.

Considera-se que tanto a formação de sómitos como o relógio molecular são processos independentes das estruturas axiais do embrião, a notocorda e o tubo neural, e das moléculas sinalizadoras aí produzidas, nomeadamente *Sonic hedgehog* (Shh). Esta faz parte da família de proteínas *Hedgehog* envolvida na regulação de diversos processos embrionários, mas que nunca

foi implicada na somitogénese. Contudo, experiências usando quimeras codorniz/galinha indicam que as estruturas axiais regulam a formação bilateral e simétrica dos sómitos. Na verdade, nos ratinhos mutantes para Shh a coluna vertebral está ausente, apresentando apenas cinco a seis costelas.

Com este trabalho pretendeu-se estudar o papel de Shh proveniente das estruturas axiais na regulação da somitogénese. A análise da expressão dos receptores de Shh *smoothened* e *patched* indica que a MPS está apta para responder à sinalização de Shh vinda da notocorda. Após remoção da notocorda, verificou-se um atraso na formação periódica de sómitos, que foi acompanhado por um aumento na periodicidade das oscilações moleculares. Foi possível recapitular estas alterações usando inibidores de Shh e restaurá-las com a adição de Shh, sugerindo que esta molécula é responsável pelas perturbações observadas após ablação da notocorda. Verifica-se que a periodicidade de formação de sómitos é recuperada ao longo do tempo, ao mesmo tempo que se observa uma sobre-expressão de *raldh2*. De facto, a adição de AR exógeno permite a recuperação da formação de sómitos. Na ausência de Shh, há um aumento da expressão na PSM dos seus efectores moleculares, os Glis, na sua forma repressora e pensa-se que o AR inibe a sua actividade. O papel de Shh na especificação da MPS M e L foi também avaliado. Verificámos a existência de um sinal difusível que percorre a MPS anterior ao longo do seu eixo M-L e mostramos que Shh é responsável pelo recrutamento de células laterais da MPS para integrar o sómito em formação. Para além disso, o uso de quimeras codorniz/galinha permitiu verificar que o território L da MPS prospectiva adquire um destino mediano quando posicionado na região M prospectiva, o que provavelmente se deve também a Shh. Apresentamos um modelo explicativo da actividade de Shh durante a especificação da MPS e na somitogénese e também da sua interacção com outras moléculas sinalizadoras que actuam na MPS. Os resultados aqui apresentados levam-nos a concluir que Shh é um componente adicional da complexa rede molecular subjacente ao controlo temporal da formação de sómitos, implicando esta via de sinalização na somitogénese pela primeira vez.

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# ABBREVIATIONS LIST

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| | |
|---|---|
| **AER** | apical ectodermal ridge |
| **A-P** | anterior-posterior |
| **Aph** | axial paraxial hinge |
| **BMP** | bone morphogenetic protein |
| **CiA** | cubitus interruptus activator |
| **CiR** | cubitus interruptus repressor |
| **Chip** | chromatin immunoprecipitation |
| **CK1** | casein kinase 1 |
| **Cos2** | costal 2 |
| **DAB** | diaminobenzidine tetrahydrochloride |
| **Dhh** | desert hedgehog |
| **DiI** | 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate |
| **Disp1** | Dispatched 1 |
| **Dll** | delta-like |
| **DMSO** | Dimethyl sulfoxide |
| **EB** | embryoid body |
| **ECM** | extracellular matrix |
| **ERK** | extracellular-signal-regulated kinase |
| **Fgf** | Fibroblast growth factor |
| **FgfR** | Fibroblast growth factor receptor |
| **Foxa2** | forkhead box a2 |
| **Fp** | floor plate of the neural tube |
| **Fu** | fused |
| **Gas1** | growth arrest specific gene 1 |
| **GliA** | glioma activator |
| **GliR** | glioma repressor |
| **Grk2** | G-protein-coupled receptor kinase 2 |
| **GSK3β** | glycogen syntethase kinase 3β |
| **Hes** | Hairy/Enhancer-of-uplit |
| **Hh** | hedgehog |
| **HhNp** | processed, biological active N-terminal hedgehog |
| **HN** | Hensen's node |
| **Hnf3β** | hepatocyte nuclear factor 3β |
| **HSPG** | heparin sulfate proteoglycan |
| **Ihh** | indian hedgehog |
| **LDA** | ligand-dependent antagonism |
| **lfng** | lunatic fringe |
| **L-PSM** | lateral presomitic mesoderm |
| **MAPK** | mitogen-activated protein kinase |
| **MET** | mesenchymal-to-epithelial transition |
| **M-L** | medial-lateral |
| **MN** | motorneurons |

| | |
|---|---|
| **M-PSM** | medial presomitic mesoderm |
| **NICD** | notch-intracellular domain |
| **No** | notochord |
| **NT** | neural tube |
| **P-D** | proximodistal |
| **PI3K** | phosphatidylinositol-3-OH uinase |
| **PKA** | protein kinase A |
| **PL-PSM** | prospective lateral presomitic mesoderm |
| **PM-PSM** | prospective medial presomitic mesoderm |
| **PSM** | presomitic mesoderm |
| **Psn** | presinilin |
| **Ptch** | Patched |
| **qPCR** | quantitative polymerase chain reaction |
| **RA** | retinoic acid |
| **Raldh2** | retinaldehyde dehydrogenase 2 |
| **RAR** | retinoic acid receptor |
| **RARE** | retinoic acid response element |
| **R-C** | rostral-caudal |
| **Shh** | sonic hedgehog |
| **siRNA** | small *i*nterfering RNA |
| **Skn** | skinny hedgehog |
| **Smo** | smoothened |
| **Spry2** | sprouty 2 |
| **SSD** | sterol-sensing domain |
| **Sufu** | suppressor of fused |
| **V** | ventral interneuron |
| **VAD** | vitamin A deficient |
| **vt** | wnt3a hypomorphic mutant vestigial tail |
| **Wnt** | Wingless-wint |
| **ZPA** | zone of polarizing activity |

# CHAPTER1.
# GENERAL INTRODUCTION

# 1. VERTEBRATE EMBRYO DEVELOPMENT

A segmented body axis is characteristic of all vertebrate species, easily observed in the vertebrate column and its associated components: vertebrae, intervertebral discs, axial muscles, dorsal root ganglia and blood vessels. The formation of these skeletal functional units provides a high degree of motility to the adult body and constitutes an efficient protection to the internal organs. The process of segmentation is initiated early in the developing embryo through the formation of repeated segments, the somites, along the anterior-posterior (A-P) body axis. Somites are blocks of cells formed from the anterior end of the mesenchymal presomitic mesoderm (PSM) that have a key role in the subsequent patterning of the body giving rise to all segmented structures in the adult body, such as vertebrae, intervertebral disks and ribs, the dermis of the back and body skeletal muscles, except those of the head.

## 1.1 EARLY STEPS IN VERTEBRATE DEVELOPMENT

Presomitic mesoderm formation occurs during gastrulation, in which a series of cellular rearrangements take place to organize the three embryonic germinative layers: ectoderm, mesoderm and endoderm. Gastrulation is a key morphogenetic process that in the chick and mammalian embryos from cells that migrate through the primitive streak, which starts as a posterior thickening of the epiblast. As cells migrate towards the primitive streak, it elongates towards the future anterior region of the embryo and defines all embryo axes: posterior-anterior, dorsal-ventral and left-right. At the anterior end of the fully extended primitive streak it is possible to observe a cellular thickening designated Hensen´s node (HN), which constitutes the embryonic organizer. In the chick, this corresponds to the developmental stage 4 Hamburger and Hamilton (HH) (Hamburger and Hamilton, 1992) (Fig. 1.1). As soon as the primitive streak is formed, epiblast cells migrate through it and adopt distinct fates: cells ingressing earlier will be positioned more anteriorly than cells migrating later in development. For example, the prospective head mesoderm ingresses earlier than the presumptive somitic mesoderm. While cell ingression continues, the HN starts to regress towards a more posterior position, concomitant with the formation of the axial structures. As it moves posteriorly, anterior structures are laid down while behind gastrulation is still taking place. As a consequence, avian and mammalian embryos display a clear A-P gradient of developmental maturity (Fig. 1.1). In the chick embryo, primitive streak regression is completed around 16-somite stage. From 16-20 somite stages, new mesodermal cells

contributing to more caudal fates arise from the tail bud, a mass of highly packed undifferentiated cells which corresponds to a functional remnant of the primitive streak (Schoenwolf, 1978; Schoenwolf et al., 1985; Catala et al., 1995). A detailed analysis of cell movement at stage 4HH (fully extended HN) has shown that cell ingression occurs in response to chemotactic signals belonging to the Fibroblast growth factor (Fgf) family: cells are attracted to Fgf4 present in the anterior-most portion of the streak and repealed by Fgf8 from the posterior region (Yang et al., 2002).

Using cell-labelling and lineage-tracing techniques, several studies have localized the PSM precursor cells (P-PSM) in the early chick embryo. In the epiblast (stage HH3), these prospective PSM cells are located bilateral to the midline while later in development, they are located in the anterior region of the primitive streak (stage HH4) and then in the tailbud (Selleck and Stern, 1991; Schoenwolf et al., 1992; Hatada and Stern, 1994; Catala et al., 1996; Psychoyos and Stern, 1996; Sawada and Aoyama, 1999; Freitas et al., 2001). As the HN regresses, these presumptive PSM cells will ingress and form two rows of mesenchymal mesoderme on both sides of the midline structures that are also being laid down. A correlation between the localization of the P-PSM cells along the A-P axis of the streak and their final mediolateral (M-L) position in the PSM can be observed: presumptive cells located more anteriorly contribute to the medial portion of PSM and somites, whereas more posterior regions originate the lateral PSM and somites (Selleck and Stern, 1991; Schoenwolf et al., 1992; Psychoyos and Stern, 1996; Sawada and Aoyama, 1999; Freitas et al., 2001; Eloy-Trinquet and Nicolas, 2002b; Iimura et al., 2007) (Fig. 1.1). Therefore, medial and lateral portions of both the PSM and somites are produced at distinct times by the primitive streak. Furthermore, grafting experiments of the P-PSM cells have shown that the prospective medial PSM territory exhibits stem cell behaviour, being able to contribute to the formation of all axial levels of the PSM (Selleck and Stern, 1991; Iimura et al., 2007). Similarly, it has been proposed that in the mouse embryo somites derive from a stem cell niche located in the primitive streak (Nicolas et al., 1996; Cambray and Wilson, 2007) in which anterior and posterior domains contribute to medial and lateral somitic cells, respectively (Eloy-Trinquet and Nicolas, 2002a).

These early morphogenetic processes in vertebrate body formation are crucial for the correct organization of the adult body and have been thoroughly studied. We now know that the final position of PSM cells in the embryo depends both on the time at which they are produced (which specifies their A-P position) and on their location along the primitive streak A-P axis, which determines their position along the M-L embryo axis. However, P-PSM cells in the

primitive streak are not completely committed and their fate can be changed if grafted into a different A-P position (Garcia-Martinez and Schoenwolf, 1992; Garcia-Martinez et al., 1997). Interestingly, this plasticity is higher in 'younger' caudal mesodermal cells, reflecting the A-P gradient of developmental maturity (Garcia-Martinez and Schoenwolf, 1992).

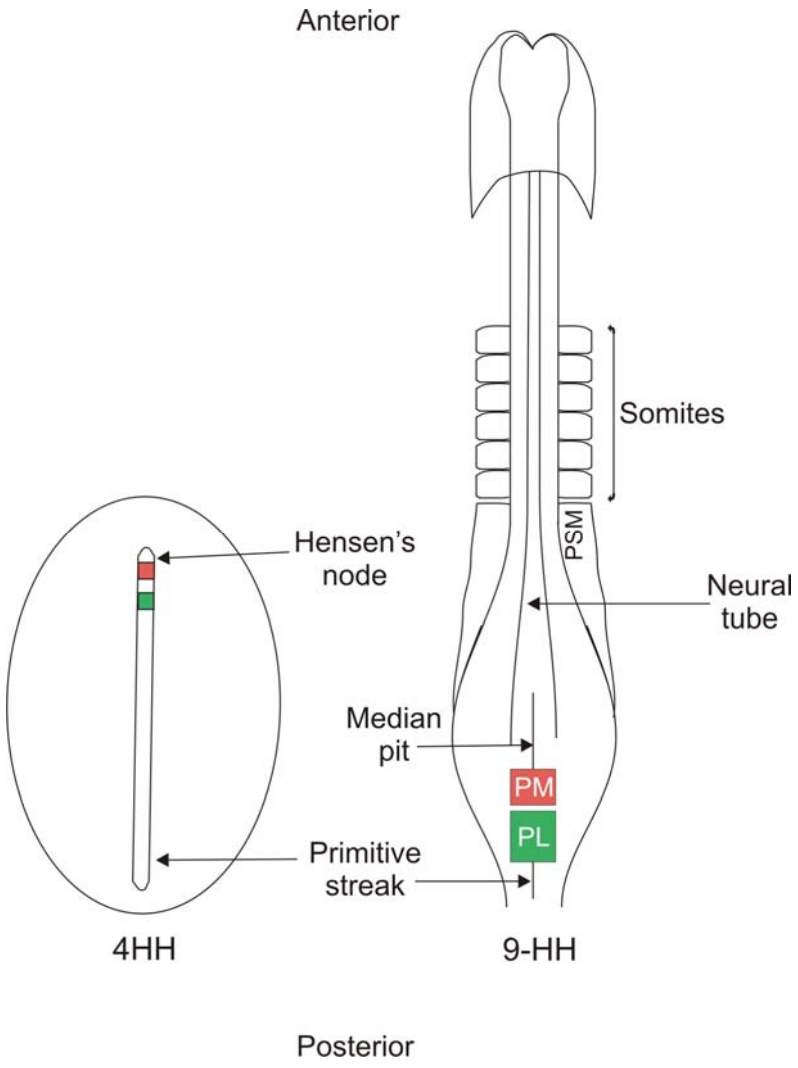


**Figure 1.1. Different stages of chick embryo development.** Shematical representation of two distinct chick development stages, 4HH and 9HH (Hamburguer and Hamilton, 1951) in a dorsal view. The embryo Hensen's node is visible at the anterior end of the primitive streak at stage 4HH. As the Hensen's node regresses caudally, anterior structures are laid down, whereas gastrulation is still occurring in the posterior part of the embryo. Consequently, a clear anterior-posterior gradient of differentiation is observed, as depicted in the 9-HH embryo. Somites are formed every 90 minutes from the anterior portion of the presomitic mesoderm (PSM) and flank the axial neural tube. The localization of the prospective medial (PM, pink) and lateral (PL, green) territories is indicated in both stages.

## 1.2 SEGMENTATION OF THE VERTEBRATE BODY

The process of segmentation is initiated early in development and involves the formation of somites along the A-P embryonic axis. Somites are formed in pairs from the rostral portion of the PSM, a strip of mesenchymal tissue that flanks the axial organs notochord and neural tube. The developmental stages of the chick embryo have been meticulously characterized by Hamburguer and Hamilton (Hamburger and Hamilton, 1992), whose classification system includs several morphological features including the number of somite pair formed. Somites have also been classified by Roman numbers according to their maturity along the A-P axis: the forming somite is considered S0 and the following presumptive ones are denoted in negative Roman numerals (S-I, S-II, *etc*) (Pourquie and Tam, 2001) whereas segmented somites are labeled with positive Roman numerals, with SI being the most recently formed somite (Christ and Ordahl, 1995) (Fig. 1.2A). Concomitant with somitogenesis, the embryo is elongating due to the continuous contribution of new cells from the tail bud region and this occurs until the final number of somites is reached. Both the total number of somites formed and the periodicity with which they are formed are species-specific parameters. It is classically considered that in the chick embryo, a new pair of somites is formed every 90 minute while in the mouse and zebrafish embryos a pair of somites buds off from the PSM every 120 and 30 minutes, respectively. However, variations in this periodicity have been observed both in mouse and chick embryos, depending on the axial position. In mouse, it has been shown that the first somites are formed faster and the last ones slower than the considered 120 minutes (Tam, 1981) whereas in the chick, formation of the last somites might require ~150 minutes (Tenin et al., 2010).

For many years, several unsuccessfully attempts have been made to disturb the PSM segmentation schedule. Experimental manipulation of the PSM, including ablations at distinct A-P levels and heterotopic or orthotopic transplantations even after inversion of the piece's A-P orientation did not disturb the original sequence of somite formation (Packard, 1978; Stern and Bellairs, 1984). These results indicate that PSM cells have an intrinsic ability to segment, which was probably acquired at their migration through the primitive streak (Christ et al., 1998).

Similarly to what occurs in the PSM, there is a sequence of somite maturation as they occupy more rostral positions in the embryo. This maturation leads to the compartmentalization of the somitic cells and subsequent formation of the dermis, skeletal muscle, vertebral elements and blood vessels. Somite compartmentalization imposes an equally segmented organization of non-

somitic derivatives such as neural crest cell migration, motor axons' trail and the pattern of the dorsal root ganglia development (reviewed in Christ and Ordahl, 1995).

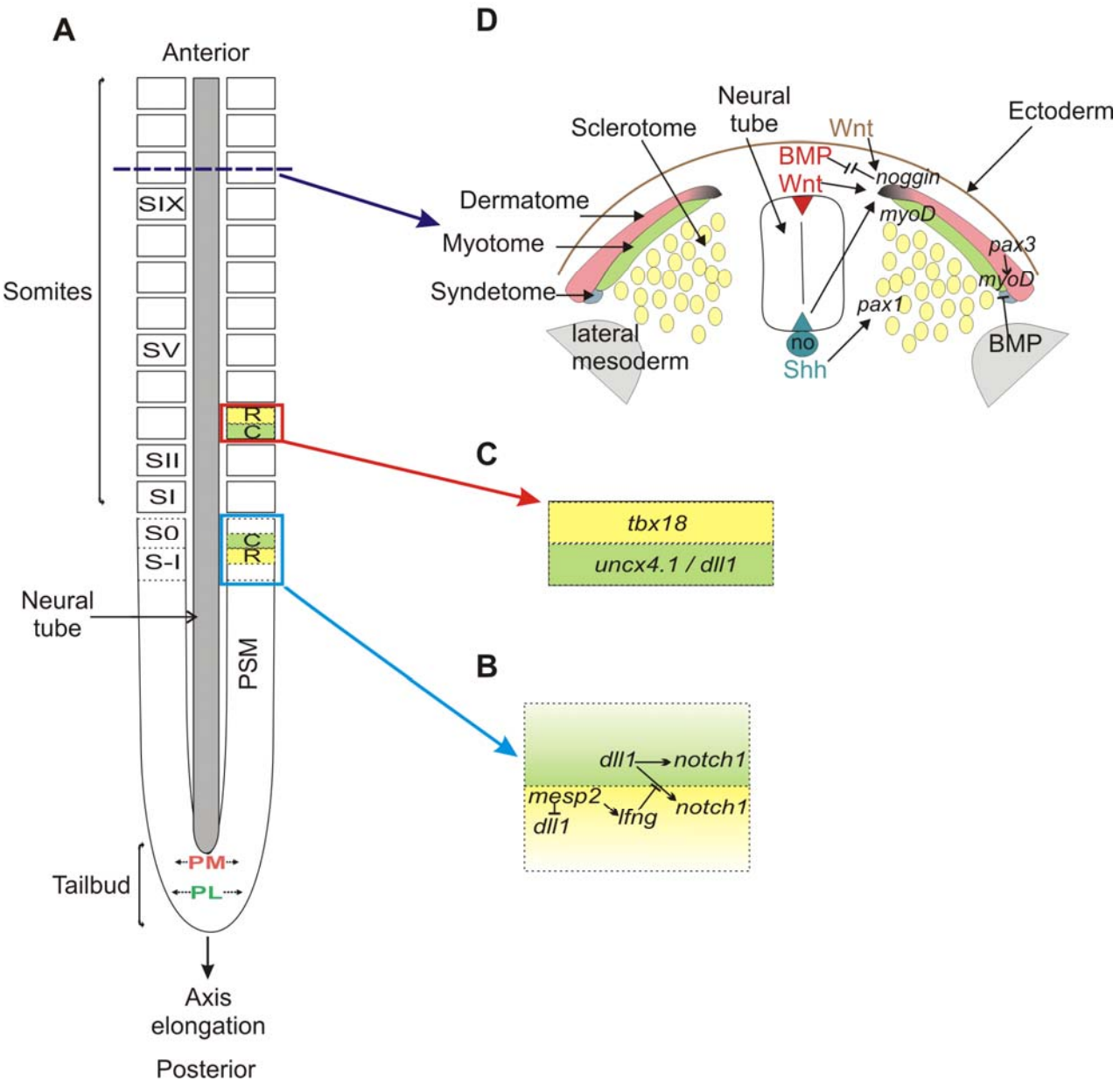


**Figure 1.2. Embryo segmentation and somite patterning. (A)** Schematical representation of the caudal portion of a chick embryo during somitogenesis. Cells from the embryo tailbud are added to the presomitic mesoderm (PSM), leading to the posterior elongation of the embryonic axis. Prospective medial cells (PM) contribute to the medial-most PSM while presumptive lateral ones (PL) originate the lateral PSM (arrows indicate future cell position). Somites are classified according to their degree of maturation: segmented somites are designated with positive Roman numerals (SI, SII, etc); the next somite to be formed is S0 and the subsequent prospective ones are labeled with negative Roman numerals (S-I, etc) (Christ and Ordahl, 1995; Pourquie and Tam, 2001). **(B)** In the anterior PSM, somite polarity genes are expressed in stripes, defining the rostral (R) and caudal (C) portion of the future somites. On the prospective

C somite domain, *dll1* expression activates *notch* while in the presumptive R portion, *mesp2* inhibits *dll1* and induces *lfng*, which inhibits *notch* in this compartment (Takahashi et al., 2000; Morimoto et al., 2005). The confrontation of Notch- activated and –inactivated domains define a somitic boundary. **(C)** Epithelial somites present distinct specific gene expression in their R and C compartments. *Uncx4.1* and *dll1* are expressed in the C somitic domain (Bettenhausen et al., 1995; Mansouri et al., 1997; Palmeirim et al., 1998; Schragle et al., 2004) while *tbx18* is expressed in the R domain (Kraus et al., 2001; Haenig and Kispert, 2004; Tanaka and Tickle, 2004). **(D)** Somite differentiation starts a few hours after budding from the PSM. Patterning of the somite by Sonic hedgehog (Shh) from the notochord (no) and neural tube floor plate (blue triangle), Wnt from the overlying ectoderm (light borwn) and neural tube roof plate (red triangle) and BMP from the roof plate and lateral mesoderm induce somite differentiation into its distinct compartments. The dermamyotome is subdivided into the dermatome (that will form the dermis of the back, where *pax3* is expressed), the myotome (originates the skeleton muscles; expresses *myoD*) and the syndetome (gives rise to the tendons) (Brent et al., 2003). *Noggin* in the dorsomedial somite antagonizes BMP to ensure a myogenic differentiation. Sclerotomal cells will originate all the skeleton elements. Shh induces *pax1* expression in the sclerotome and counteracts BMP effect. See text for further detail. For a review, see Stockdale et al., 2000.

## 1.3 MECHANISMS OF SOMITE FORMATION

During somitogenesis the posterior PSM is continuous replenished by newly formed mesenchymal cells that gradually mature as they become progressively displaced anteriorly until they are integrated in somites. In fact, a metameric arrangement of cells while still within the PSM has been visualized by stereoscanning electron microscopy (Meier, 1979; Packard, 1980; Meier and Jacobson, 1982; Packard and Meier, 1983). In the chick and quail embryos, 10-12 metameric cell clusters, designated somitomeres, have been described (Packard, 1980; Packard and Meier, 1983). However cells do not simply leave the node and make their way orderly until somite integration. Cellular movement within the PSM has been analyzed by following cells either labeled with the vital dye 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) or their descendants after electroporation with fluorescent markers, showing that cells disperse along the PSM and often change neighbors, slowing down only when they localize within four to five somites from the anterior PSM tip (Kulesa and Fraser, 2002; Benazeraf et al., 2010). Prior to somite formation, the most rostral PSM cells become packed and polarized, preparing for the mesenchymal-to-epithelial (MET) transition necessary for somite formation. Somite formation is not a straight-forward event and involves a series of complex cellular rearrangements. In the chick embryo, PSM epithelialization starts from the medial-most cells that elongate and recruit neighbor ones until the somite finally pulls apart from the PSM through a ball-and-socket mechanism

(Kulesa and Fraser, 2002; Martins et al., 2009). Somites thus formed have an epithelial layer of cells, which are still able to move and trade places with the mesenchymal cells of the somite lumen (Martins et al., 2009).

Somite cell compaction is accompanied by arrangements in the extracellular matrix (ECM) molecules, which have been implicated in somite formation and epithelialization. In fibronectin null mouse embryos, even though paraxial mesoderm is formed, no morphological distinguishable somites are produced (George et al., 1993; Georges-Labouesse et al., 1996; Yang et al., 1999). In fact, for proper somitogenesis to occur, the PSM needs to be surrounded by an intact fibronectin matrix (Duband et al., 1987; Rifes et al., 2007; Martins et al., 2009). Previous reports show that morphological somite formation requires the presence of the overlying ectoderm (Palmeirim et al., 1998) and in fact the role of ectoderm is to provide the fibronectin protein (Rifes et al., 2007). The dominant-negative mutation of the protocadherin-encoding gene *papc* disrupts the epithelial organization of the segment border's cells (Rhee et al., 2003). However, *papc*-null mice show no segmentation defects (Yamamoto et al., 2000), suggesting that segment border formation and epithelialization are independent processes. In fact, *paraxis*-null embryos present segmented somites but the newly formed ones are not completely epithelialized (Burgess et al., 1996). In these mutants, the axial skeleton and the skeletal muscles still formed but were improperly patterned. Furthermore, other molecules belonging to the cadherin and Eph–Ephrin families of proteins have also been implicated in the paraxial mesoderm MET and somite formation (Duband et al., 1987; Barrios et al., 2003).

The role of the PSM surrounding structures in somite formation has been addressed by several studies. By performing embryo explant culture differently delimited to include either the notochord, neural tube or both, Packard and collaborators described somite formation to be independent of the axial structures (Packard and Jacobson, 1976; Packard, 1980). However, a report from the same authors shows that when a quail PSM is grafted into a chick embryo, the inserted tissue progressively adjusts the location of each intersomitic boundary to that of the host contralateral part (Packard and Meier, 1983), strongly suggesting that a signal coming from the embryo midline structures may be controlling PSM somite formation. The axial structures have been implicated in the viability and differentiation of the somitic cells. Isolation of the PSM from the midline structures leads to an increased cell death and disappearance of sclerorome and myotome somitic compartments and the tissues normally differentiating from them – cartilage and dorsal muscles (Rong et al., 1992). This was later shown to be mediated by Shh as grafting of Shh expressing quail fibroblasts mimicked the survival action of the axial organs (Teillet et al., 1998).

## 1.4 SOMITE POLARITY

The future somite begins to be subdivided in rostral and caudal halves even before emerging from the PSM, by differential somitic polarity gene expression restricted to each half of the presumptive somite. Therefore, molecular segmentation occurs prior to morphological segmentation (Palmeirim et al., 1998). The mechanisms leading to rostral-caudal (R-C) polarity are mainly dependent on Mesp2 and Notch/Dll signaling and their reciprocal regulation (reviewed in Saga, 2007). *Delta-like1* (*dll1*), which encodes a Notch ligand, *notch* membrane receptor and *uncx4.1*, a paired-type homeobox transcription factor, are expressed in stripes in the anterior PSM, confering a posterior fate to prospective somites in chick (Palmeirim et al., 1998; Schragle et al., 2004) and mouse embryos (Bettenhausen et al., 1995; Mansouri et al., 1997). On the other hand, genes like *mesp2* (*meso1* in chick), which encodes a basic helix-loop-helix (BHLH) transcription factor and *delta-like3* (*dll3*) are expressed in the presumptive anterior somite domain (Dunwoodie et al., 1997; Saga et al., 1997; Buchberger et al., 1998). *Mesp2* expression in the presumptive anterior somite domain inhibits *dll1* expression while inducing *lunatic fringe* (*lfng*), which inhibits Notch activity in this compartment (Takahashi et al., 2000; Morimoto et al., 2005). On the prospective posterior somite domain, *mesp2* is absent and *dll1* is expressed and therefore Notch is activated. Thus, somite boundaries are formed when cells with high *mesp2* and low Notch signaling levels are confronted with cells in the opposite state – high Notch and low *mesp2* expression, suggesting the existence of a feedback loop between both molecules (Fig. 1.2B). The correct establishment of the R-C somite polarity is crucial for subsequent differentiation. *Mesp2*-null mutants, for example, lack segment borders and present proximal fusion of the ribs (Saga et al., 1997). The maintenance of rostral and caudal identities in newly formed somites depends on *tbx18* and *uncx4.1* expression, respectively (Fig. 1.2C). *Tbx18* is a transcription factor containing a conserved T-box DNA binding domain expressed as two stripes in the anterior PSM and in the anterior somitic compartment of mouse (Kraus et al., 2001) and chick embryos (Haenig and Kispert, 2004; Tanaka and Tickle, 2004). Mouse mutants for *tbx18* establish a correct somite A-P polarity but are not able to maintain it because cells from the caudal somitic compartment are constantly invading the anterior domain (Bussen et al., 2004). In the chick, *tbx18* seems also to play a role in somite boundary formation: transplantation of a *tbx18*-expressing tissue to unsegmented PSM normally not expressing that gene induces the formation of an ectopic somitic boundary, suggesting that the interface between *tbx18*- expressing and non-expressing cells defines a somitic boundary (Tanaka and Tickle, 2004).

## 1.5 SOMITE DIFFERENTIATION

Recently formed epithelial somites (somites I-III) do not present any sign of differentiation. In fact, if these somites are rotated, inverting their R-C orientation or if the dorsal half of a somite is replaced by a ventral half, the expected somite derivatives are formed (Dockter and Ordahl, 2000). Similarly, switch-grafts of the medial and lateral portions of newly formed somites have shown that these are interchangeable (Ordahl and Le Douarin, 1992). These results indicate that newly formed somites are still plastic and that their developmental fate is position dependent and determined by external signals. A few hours after budding off from the PSM, somitic compartments start to differentiate due to the influence of signals from surrounding structures: ectoderm, lateral plate, notochord and neural tube. Patterning of the somites is crucial for further differentiation and mainly involves interactors of three signaling pathways: Sonic Hedgehog (Shh), a morphogen produced by the notochord and floor plate of the neural tube, Wingless (Wnt) signaling from the dorsal neural tube and ectoderm and Bone morphogenetic protein (BMP) coming from the dorsal neural tube and lateral plate mesoderm (reviewed in Stockdale et al., 2000). Somite differentiation involves an epithelial-to-mesenchymal transition occurring in the ventral portion of the somite to form the sclerotome. These mesenchymal cells migrate to surround the notochord and constitute the precursors of cartilage and bone, giving rise to the axial skeleton (vertebrae and intervertebral disks) and the ribs. The dorsal portion of the somite retains its epithelial characteristics and constitutes the dermamyotome, which will form skeletal muscles, dermis and vascular derivatives (reviewed in Stockdale et al., 2000). Along the M-L axis, the somite can be further subdivided into medial and lateral compartments (summarized in Fig. 1.2D).

The dermamyotome is responsible for the formation of the dermis of the back (from its dermatome compartment), skeletal muscle (from the myotome subdivision) and also tendon progenitors (from the syndetome, which is established and maintained by both the sclerotome and myotome) (Brent et al., 2003; reviewed in Stockdale et al., 2000). The medial portion of the myotome is the source of the epaxial muscles, the deep back muscles that surround the axial body skeleton while the lateral section contributes to the hypaxial musculature of the limbs, abdomen as well as the ribs (Ordahl and Le Douarin, 1992; Olivera-Martinez et al., 2000). *Pax3*, a member of the *pax* family of paired-type homeodomain genes, is firstly expressed in the PSM and epithelial somites and later becomes restricted to the dermamyotome, where together with *pax7* define the myogenic precursors that will constitute the myotome (reviewed in Stockdale et al., 2000). Shh and Wnt signaling act in concert to stimulate myogenesis by initiating and sustaining the

expression of myogenic regulatory factors such *myoD* and *myf5* and *noggin* expression in the dorsomedial somite (Hirsinger et al., 1997; Maroto et al., 1997; Borycki et al., 1998). BMP signaling from the neural tube is opposed by *noggin* in mediolateral cells while lateral plate produced BMP induces the expression of lateral fate markers such as *sim1* and loss of *myoD/myf5* (Pourquie et al., 1995; Pourquie et al., 1996; Reshef et al., 1998), allowing these cells to migrate to their final destinations before differentiating into muscle cells (summarized in Fig. 1.2D).

Sclerotomal cells are the precursors of the axial skeleton elements. Noggin and Shh signalling are involved in initiating and sustaining the sclerotome by inducing *pax1*expression and antagonizing the effect of the dorsal BMPs and Wnt signals (reviewed in Christ et al., 2000; Stockdale et al., 2000). *Pax1*, *pax9* and the *mesenchymal forkhead1* (*mfh1*) expressed in the sclerotome are essential for cell proliferation and important for the proper specification of the sclerotome compartment. The sclerotome becomes subdivided in a rostral and a caudal half, through a process called resegmentation. Each half exhibit distinct combinations of gene expression and important developmental consequences: due to the different molecular properties of the domains, neural crest cells and motorneurons are only able to invade the rostral sclerotome compartment (reviewed in Stockdale et al., 2000). Moreover, each half segment of the sclerotome contributes to distinct elements of a vertebra: the rostral half of one somite forms the caudal portion of a vertebra while the caudal half of the adjacent somite originates the rostral portion of that same vertebra.

A functional musculoskeletal system depends entirely upon the attachments that transmit force from muscle to bone, and thus requires the coordinated development of muscle, cartilage, and tendon. The arrangement observed in somitic differentiation allows the integration of those structures in such a way as to provide the rigidity and flexibility necessary for vertebrate movement. Sclerotome resegmentation allows muscles coming from the dermamyotome to connect two adjacent vertebra of the vertebral column. Furthermore, the specification of tendon progenitors in between those two somitic compartments, supplies the required connective tissue to translate the generated force into movement.

## 1.6 AXIAL SPECIFICATION

Although somites along the early embryo A-P axis are morphologically similar, they give rise to very different vertebrae: cervical, thoracic, lumbar, sacral and caudal. This differentiation of the somites is controlled at two distinct levels. One includes the mechanisms responsible for the

formation and differentiation of the somitic compartments, discussed above. The second mechanism of control relies on positional information along the A-P axis, accounting for the genesis of morphologically distinct structures from somites located at different axial levels. Interestingly, determination of the axial identity seems to occur before cell incorporation into somites. Transplantation experiments in which unsegmented PSM from the thoracic level replaced the same tissue from the cervical region induced the formation of ectopic thoracic ribs even though they are no longer in the thoracic region (reviewed in Stockdale et al., 2000). At the molecular level, the Hox gene family, which encodes homeobox-containing transcription factors, has been shown to play a pivotal role in the control of segmental identity in the axial skeleton (reviewed in Wellik, 2007; Mallo et al., 2009). This family of genes is arranged in genomic clusters and presents spatial and temporal colinearity, meaning that Hox genes located at the 3' end of the clusters (also known as "anterior" genes) are activated earlier and specify more anterior structures whereas those located towards the 5' end of the cluster ("posterior" Hox) are expressed later in development and induce more posterior identities. Accordingly, both ectopic expression and inactivation of Hox gene expression in mice results in vertebra identity transformations, which varied depending on the specific Hox gene or genes affected (reviewed in Mallo et al., 2009; Aulehla and Pourquie, 2010). It has been shown that the Hox genes are collinearly expressed in the epiblast PSM precursors before ingression through the primitive streak and that overexpression of more posterior Hox genes delays the timing of cell ingression (Iimura and Pourquie, 2006). In fact, it has also been proposed that it is the confrontation with an unknown signal from the embryo organizer center that stabilizes the cell Hox code of expression (Wacker et al., 2004). However, the mechanisms by which Hox genes control vertebral identities and the interactions among the different Hox are not very well understood. Vertebral identity definition probably results from the combinatorial of the signaling molecules acting throughout the PSM or even earlier during development and the specific Hox genes expressed at each particular axial level.

## 1.7 MODELS ACCOUNTING FOR PERIODIC SOMITE FORMATION

The striking periodicity of somite formation has always intrigued embryologists. To explain this remarkable timely regulated mechanism, several models have been proposed, which include the cell cycle model, the Meinhardt's model and the clock-and-wavefront model, briefly discussed below.

The cell cycle model was postulated based on the observation that embryos exposed to a single heat-shock treatment presented several segmental abnormalities (Primmett et al., 1989). These were observed along the embryonic A-P axis every 6-7 segments on average in the chick embryo (Primmett et al., 1988; Primmett et al., 1989) and each 5 somites in zebrafish (Roy et al., 1999). In the chick, the formation of 6-7 somites takes 9-10 hours, which corresponds to the time required for the completion of the cell cycle in the PSM (Primmett et al., 1989). In zebrafish, however, this correlation was never attained (Roy et al., 1999). Moreover, the exposure of the chick PSM tissue to cell cycle chemical inhibitors resulted in comparable segmental anomalies, which reinforced the role of the cell cycle in the segmentation process (Primmett et al., 1989). This model was further supported by mathematical modulation (Collier et al., 2000; McInerney et al., 2004). It postulated that cells from the anterior-most PSM present some degree of cell cycle synchrony: cells were in one of two states (P1 or P2) within the mitotic phase and the time interval between these two states was 90min. Cells arriving first at the P2 state would recruit P1 cells to incorporate the next forming somite (Primmett et al., 1989).

Meinhardt proposed that the metameric pattern is produced by confrontation of groups of cells with different characteristics. Cells composing a somite first oscillate between two distinct states A and P before obtaining their final identity and a physical barrier forms between these two groups of cells (Meinhardt, 1986). A problem arising from this model is that a barrier can also be formed in the middle of a somite and to overcome this, Meinhardt postulated a third cell state, the segment border (S) and thus a somite would arise at each P-S confrontation (Meinhardt, 1986). However, even though a subdivision of A and P somitic compartments can be molecularly identified (Palmeirim et al., 1998; Schragle et al., 2004; Tanaka and Tickle, 2004), no S cells have been identified so far. Additionally, it has been shown that during somite formation cells are able to intermingle between caudal and rostral compartments of two consecutive somites (Kulesa and Fraser, 2002; Martins et al., 2009).

The theoretical Clock-and-Wavefront model was proposed after the observation that reducing the number of cells in a frog blastula leads to the formation of a smaller embryo with a normal somite number (Cooke and Zeeman, 1976). The model proposes the existence of two independent phenomena occurring in the PSM and accounting for the periodic somite formation. PSM cells possess an intrinsic biochemical oscillator, a clock, and neighbor PSM cells oscillate synchronously between a permissive and a non-permissive state of somite formation. The second component of the model is the wavefront, which corresponds to a maturation front traveling along the embryonic A-P axis and moving posteriorly in concert with the A-P differentiation gradient of

the embryo. Thus, in order to form a somite, a group of PSM cells in the permissive state of the clock must be reached by the wavefront of differentiation (Cooke and Zeeman, 1976). Remarkably, experimental data obtained to date support both assumptions of the Clock-and-Wavefront model.

## 1.8 THE SOMITOGENESIS MOLECULAR CLOCK

The existence of an intrinsic oscillator associated to the PSM segmentation was first recognized in the chick embryo. It was observed that the mRNA coding for the bHLH transcription repressor *hairy1*, the avian homolog of the fly pair rule gene *hairy* of the Hairy/Enhancer-of-split (Hes) family, displayed very different patterns of expression in the PSM within embryos in the same development HH stage (Palmeirim et al., 1997). By dividing the embryo in half and performing explant culture in which one of the explants was immediately fixed and its contralateral was incubated for different time periods, the authors demonstrated that this dynamic expression is cyclic, being reiterated every 90 minutes, which corresponds to the time required to form a new pair of somites in the chick (Palmeirim et al., 1997). Each cycle of expression can be subdivided into three distinct phases (see Fig. 1.3A): in phase I, *hairy1* is detected in a large caudal domain expanding over about 70% of the posterior PSM and in the anterior PSM as a narrow stripe in the future posterior portion of the forming somite (S0). In phase II, the anterior stripe is maintained but the caudal expression disappears and *hairy1* transcripts appear more anteriorly in the PSM, at the 40-80% level of PSM extension (considering the tailbud as 0% extension). In phase III, when S0 is almost formed, *hairy1* expression is shifted to the anterior PSM, corresponding to about one somite-length (80-90% level of PSM extension). At the same time, a new caudal domain arises, which corresponds to the phase I of the next cycle of expression. By performing a series of elegant microsurgical experiments, the authors showed that these oscillations are an intrinsic property of the PSM cells and that the observed wave of *hairy1* expression is independent of cell movement and does not result from a posterior signal travelling along the PSM to activate the gene in anterior positions. Rather, along the PSM A-P axis, cells expressing *hairy1* are slightly out-of-phase relatively to each other, generating a kinematic wave that sweeps the PSM (Palmeirim et al., 1997). This was later confirmed by real time bioluminescence imaging in mouse embryo, which allows the analyses of cycling gene expression in a real time culture system (Masamizu et al., 2006). Using transgenic mice carrying a luciferase reporter driven by the *hes1* promoter, it was possible to observe that waves of *hes1* transcriptional

activation are propagated from caudal PSM to S0, where they are briefly stabilized before disappearing concomitantly with somite formation.

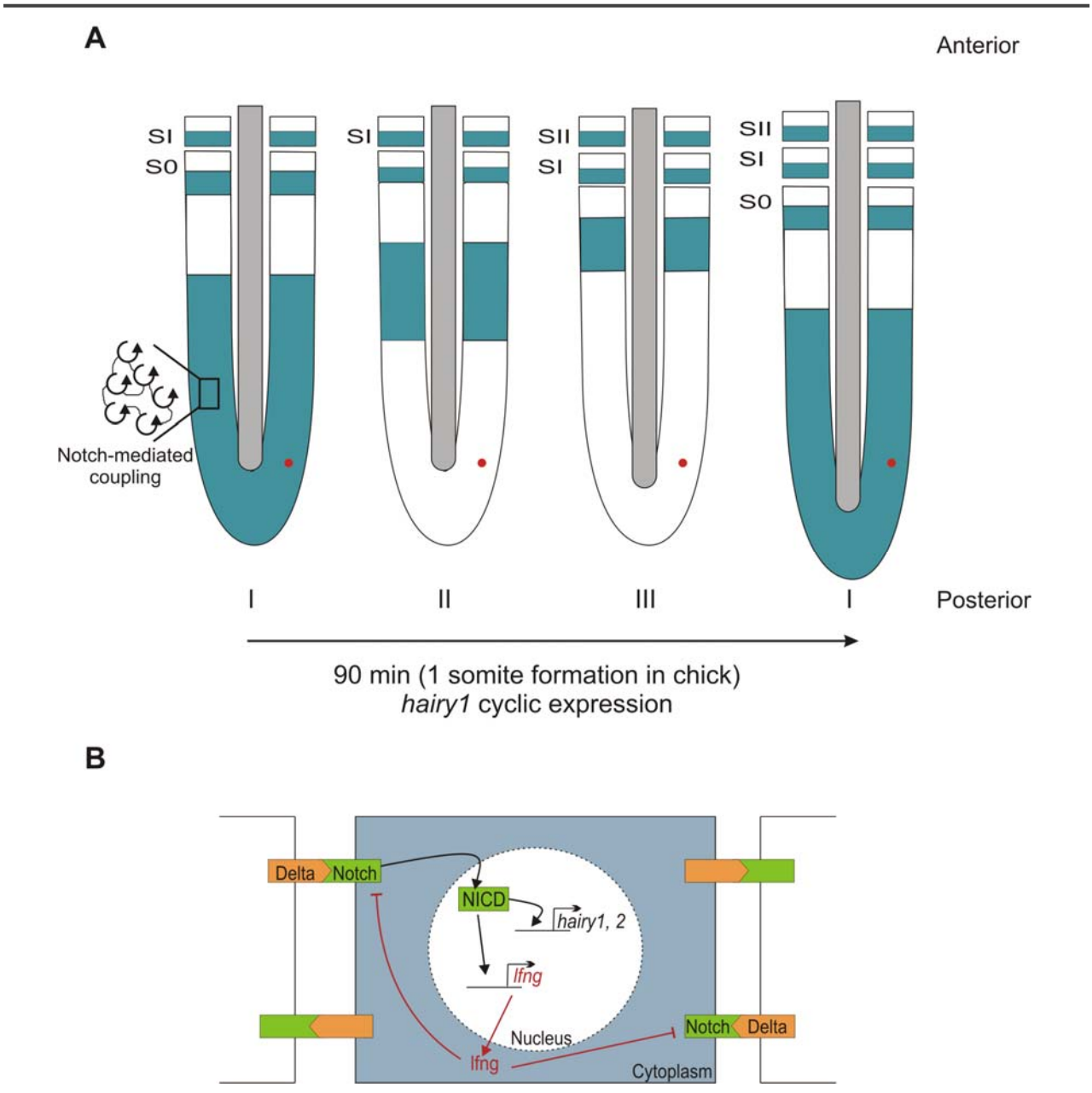


**Figure 1.3. Molecular network of the chick segmentation clock.** Schematical representation of the distinct phases of expression (I, II and III) of the chick oscillating gene *hairy1* (Palmeirim et al., 1997). *Hairy1* mRNA transcriptional oscillations are propagated as a posterior-anterior kinematic wave that sweeps the PSM and culminates with the formation of a new pair of somites (SI). This cyclic expression along the PSM is reiterated every 90min in the chick, corresponding to the time interval required for somite formation. During each cycle of expression, individual cells (represented as a red dot in the PSM) periodically turn on and off the gene. Individual cell cyclic expression in the PSM is thought to be synchronized by cell-cell coupling mediated by the Notch signaling pathway (black spiral

symbol). S0 represents the forming somite, SI and SII the two most recent formed somites. **(B)** Model representing the intercellular coupling achieved by Delta/Notch communication and the feedback loop underlying the generation of *lfng* oscillations in the chick embryo. The Delta ligand binds to the Notch receptor of adjacent cells activating the Notch signaling cascade. Upon ligand binding, the Notch intracellular domain (NCID) is released and translocated into the nucleus, where in chick activates the transcription of *hairy1*, *hairy2* and *lfng* genes (black arrows). Lfng protein modifies Notch making it less sensitive to activation by Delta (red arrows). This effect is transient due to the short life of Lfng. Oscillations are thus generated by alternation between activation of *lfng* expression and repression of Notch by Lfng (Dale et al., 2003).

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After the characterization of *hairy1* cyclic expression in the chick, many other genes were described to have a similar oscillatory behaviour. These genes are referred to as cyclic genes and constitute the somitogenesis molecular clock, suggesting that periodic somite formation is controlled by a highly regulated genetic network. It is now evident that the molecular events underlying somitogenesis are highly conserved among vertebrates, since periodic gene transcription has also been described in other animal models used in Developmental Biology - mouse, zebrafish, frog and medaka (reviewed in Andrade et al., 2007).

This rhythmic expression begins as early as gastrulation, where the clock genes *hairy1*, *hairy2* and *lunatic fringe* (*lfng*) present an oscillatory expression in the PSM precursor's cells (Freitas et al., 2001; Jouve et al., 2002). These observations suggest that the segmentation clock may be involved in defining cell fate – the number of oscillations experienced by a cell may correlate with its future localization level along the A-P body axis. A similar cyclic behaviour was observed in limb chondrogenic precursor cells, where the *hairy2* gene was shown to present an oscillatory period of 6 hours, underlying the formation of skeletal limb structures (Pascoal et al., 2007). This suggests that gene oscillations with distinct periodicity may be involved in regulating the formation of different embryonic structures and can be a widespread mechanism experienced by many cell and tissue types in the vertebrate body. In fact, work performed in Kageyama's lab reported the oscillation of *hes1* gene, homolog of the chick *hairy1*, in cultured cells such as myoblasts, fibroblasts, neuroblastoma and teratocarcinoma cells (Hirata et al., 2002). Furthermore, cyclic expression of *hes* genes has also been described in mouse neural progenitors (Shimojo et al., 2008) and in mesenchymal stem cells derived from human umbilical cord blood (William et al., 2007). Cyclic oscillations of *hes* genes in stem cells have been correlated with the maintenance of pluripotency and regulation of binary cell fate decisions, thereby generating cell type diversity (reviewed in Kageyama et al., 2007; Shimojo et al., 2008; Kobayashi et al., 2009).

The majority of the cyclic genes identified to date belong to the Notch signaling pathway (Summarized in Table 1.1). Among these are the *hes* genes, which are downstream targets of the Notch pathway and encode transcriptional repressors. These include the *hairy1*, *hairy2* and *hey2* genes in chick (Palmeirim et al., 1997; Jouve et al., 2000; Leimeister et al., 2000), *hes1*, *hes5*, *hes7* and *hey2* in mouse (Jouve et al., 2000; Leimeister et al., 2000; Bessho et al., 2001a; Dunwoodie et al., 2002) and *her1* and *her7* in zebrafish (reviewed in Holley, 2007). Furthermore, the Notch-modifying glycosyltransferase enzyme *lunatic fringe* (*lfng*) oscillates in the PSM of both mouse and chick embryos (Forsberg et al., 1998; McGrew et al., 1998; Aulehla and Johnson, 1999) and *deltaC*, a notch ligand depicts a cyclic behavior in zebrafish (Jiang et al., 2000). A microarray analysis of the mouse PSM transcriptome has identified several other genes with similar dynamic expression, belonging to the Fgf and Wnt pathways (Summarized in Table 1.1) (Dequéant et al., 2006). This study has also shown that Notch and Fgf genes oscillate in phase with each other while genes belonging to the Wnt signaling pathway are out-of-phase with Notch-Fgf.

**Table 1.1** – Genes belonging to the signaling pathways Notch, Wnt or Fgf shown to have an oscillatory behavior in mouse, chick and zebrafish embryos.

| | Mouse | Chick | Zebrafish |
|---|---|---|---|
| **Notch** | *hes1* (Jouve et al., 2000)<br>*hes5* (Dunwoodie et al., 2002)<br>*hes7* (Bessho et al., 2001)<br>*hey2* (Leimeister et al., 2000)<br>*lfng* (Forsberg et al., 1998)<br>*nkd1* (Ishikawa et al., 2004)<br>*nrarp* (Dequéant et al., 2006) | *hairy1* (Palmeirim et al., 1997)<br>*hairy2* (Jouve et al., 2000)<br>*hey2* (Leimeister et al., 2000)<br>*lfng* (McGrew et al., 1998; Aulehla and Johnson, 1999)<br>*nrarp* (Wright et al., 2009) | *deltaC* (Jiang et al., 2000)<br>*her1* (Holley et al., 2000)<br>*her7* (Oates and Ho, 2002)<br>*nrarp* (Wright et al., 2009) |
| **Wnt** | *axin2* (Aulehla et al., 2003)<br>*dact1* (Suriben et al., 2006) | | |
| **Fgf** | *dusp4* (Niwa et al., 2007)<br>*dusp6* (Dequéant et al., 2006)<br>*snail1* (Dale et al., 2006)<br>*spry2* (Dequéant et al., 2006)<br>*spry4* (Hayashi et al., 2009) | *snail2* (Dale et al., 2006) | |

## 1.9 NOTCH SIGNALING IN SEGMENTATION

The Notch signaling pathway is highly conserved along the evolutionary scale and is known to regulate cell-fate decision during embryonic development. It is activated by ligands through direct cell-cell interaction and therefore both Notch and its ligands are transmembrane proteins. The ligands binding and activating Notch are either Delta or Serrate/Jagged proteins and

ligand-receptor affinity can be modulated by posttranslational modification of the Notch extracellular domain by the glycosyltransferase Fringe, which can either potentiate or inhibit Notch signaling. Upon binding, the transmembranar domain of the receptor undergoes a proteolytic cleavage mediated by the δ-secretase activity of Presinilin 1 and 2 (Psn), releasing the Notch-intracelular domain (NICD). The NICD is translocated into the nucleus, where it associates with the DNA-binding transcription factor RBPJk, converting it into a transcriptional activator thus inducing transcription of its target genes (reviewed in Rida et al., 2004; Kageyama et al., 2007) (Fig. 1.3B).

Notch signaling seems to be crucial for vertebrate segmentation clock, since many of the known cyclic genes are Notch targets and mutations in different pathway components lead to defects in segmentation. Mice carrying mutations in any of the genes encoding ligands, receptors or downstream effectors of the pathway – *notch*, *dll1*, *dll3*, *psn1*, *lfng* and *hes7* – display segmentation defects (reviewed in Rida et al., 2004). In general, the cyclic expression is disrupted and anterior somites are formed while posterior ones are disorganized with irregularly spaced boundaries. However, *hes1*-null mutants have no somitogenesis phenotype (Jouve et al., 2000), suggesting that its function can be redundant with other *hes*-related gene. Similarly, chick embryos over-expressing *lfng* show disruption of Notch gene oscillations and perturbation of somite boundary position (Dale et al., 2003). It has been suggested that in the zebrafish embryo the role of Notch is to synchronize *her* oscillations in individual cells. Zebrafish in which the Notch signaling was affected, either by directed mutagenesis or antisense morpholinos knockdown present the same somitic defects observed in mouse: somite formation was disrupted caudally while anterior somites were formed normally (reviewed in Rida et al., 2004; Lewis et al., 2009). In these mutants, *deltaC* is still expressed in the PSM but in an uncoordinate way: a salt-and-pepper expression is observed, suggesting that cells are still oscillating individually but no longer in synchrony with their neighbours. Kawamura et al (2005) identified a new *her*-related gene (*her13.2*) that functions downstream of Fgf signaling in the PSM but independently of Notch and is required for the cyclic expression of both *her1* and *her7* genes (Kawamura et al., 2005). Thus, in zebrafish, Fgf signaling may be involved in inducing *her1*/*her7* oscillations while Notch pathway synchronizes the fluctuations of individual cells in the PSM. These observations would explain why the first somites are formed normally in mouse and zebrafish Notch mutants: after disruption of Notch signaling, cells take some cycles to drift out of synchrony. Thus, the same synchronization mechanism can be occurring in mouse since the same defects are observed after Notch signaling disruption. Accordingly, when Notch activity is completely absent as in the

double *psen1*/*psen2* knockout, which generate mutant embryos lacking all the Psen activity and thus NCID formation, dynamic gene expression is completely abolished and no somites are formed (Ferjentsik et al., 2009). The authors argue that this indicates a Notch signaling requirement for periodic gene transcription and somite formation. However, this can also be due to the complete desynchronization of PSM cells due to lack of cell-cell communication mediated by Notch.

The role of Notch signaling in cell-cell synchrony has been thoroughly analyzed, showing that Notch-intercellular coupling is essential for maintaining synchronized oscillations by reducing the internal PSM developmental noise. When PSM cells from a zebrafish embryo with continuously activated Notch signaling were transplanted to a wild-type embryo, the grafted cells caused an acceleration of *her1* oscillation in adjacent cells and consequently an anterior shift of the segment positions (Horikawa et al., 2006). This effect was found to be dependent on *deltaC* since its depletion in donor cells does not induce an anterior shift in *her1* expression. Furthermore, the authors show that following treatment with DAPT, a γ-secretase inhibitor that attenuates Notch function, *her1* mRNA levels are variable from cell to cell. Thus, the global oscillation pattern observed in the PSM seems to be maintained by Notch-intercellular communication, as demonstrated both by *in vivo* and *in silico* experiments (Horikawa et al., 2006). More or less at the same time, studies performed in the chick embryo to analyze whether cycling gene expression in the PSM is cell autonomous have shown that smaller pieces of PSM were able to maintain oscillations up to 6 hours incubation (Maroto et al., 2005). However, when posterior PSM cells were dissociated and divided in separately cultured pools, cells rapidly fell out of synchrony (Maroto et al., 2005). Using real-time bioluminescence imaging technique it was possible to follow *hes1* expression in mouse PSM for as long as 15 hours and show that dissected PSM fragments maintain stable expression but gradually become out-of-phase (Masamizu et al., 2006). These authors also followed oscillations in dissociated PSM cells showing that although they still present *hes1* oscillations, the period and amplitude of the cycles vary greatly between individual cells.

Altogether these results show that cell-cell communication is required for synchronization of PSM oscillations, which is further supported by several mathematical modeling analyses (Horikawa et al., 2006; Masamizu et al., 2006; Riedel-Kruse et al., 2007). However, these models did not take into account the dynamic cell rearrangement constantly occurring in the PSM (Kulesa and Fraser, 2002; Benazeraf et al., 2010). Recently, a theoretical model considering cell movement within the PSM shows that random cell movement promotes the synchronization of the

segmentation clock and allows synchronization to be achieved faster after an external perturbation (Uriu et al., 2010). Similarly to what has been observed in the zebrafish, the intercellular coupling in chick and mouse embryos is probably mediated by Notch signaling (see Fig. 1.3A). In fact, mouse cells otherwise unsynchronized, cultured in the presence of Dll1 protein are able to perform *hes1* mRNA and protein oscillations with a two hour periodicity (Hirata et al., 2002). Recently, it was shown that diminished Dll-Notch coupling induces an increase in somitogenesis period and somite size in zebrafish, indicating that Notch coupling controls the overall period of the segmentation clock (Herrgen et al., 2010). These observations however raise the possibility that a Notch-independent mechanism could be the generator of the cyclic gene oscillations.

## 1.10 GENERATING CYCLIC GENE EXPRESSION

The mechanism by which cell intrinsic cyclic expression is generated has been deeply analyzed and shown to rely on negative feedback regulation and short lived mRNA and proteins. *Hes* genes encode nuclear proteins that act as transcriptional repressors. They contain three conserved domains conferring them transcriptional functions: the Orange domain regulates the formation of homo- and hetero- dimers, the WRPW domain mediates transcriptional repression and acts as a polyubiquitylation signal and the bHLH domain, which contains the DNA binding site (reviewed in Kageyama et al., 2007). Most bHLH factors bind to a consensus sequence designated E box present in the promoter region of their targets, as is the case of *hes7* (Bessho et al., 2001a) but *hes1* preferentially bind to N-boxes, another consensus sequence (Sasai et al., 1992). Furthermore, Hes family members are known to negatively autoregulate their expression *via* direct binding to their own promoter (Takebayashi et al., 1994).

It has been shown that cultured cells either subjected to a serum treatment or exposed to Delta1 protein, exhibit both *hes1* mRNA and Hes1 protein oscillations with a two hour periodicity; protein oscillation is delayed ~15min compared to mRNA, probably due to the time required for protein turnover by the ubiquitin–proteasome pathway (Hirata et al., 2002). These authors showed that after induction, Hes1 protein represses mRNA transcription by directly binding to the N-box in its promoter. This repression is only transient due to the short half-life (~20min) of both *hes1* mRNA and Hes1 protein. When Hes1 protein is constitutively activated either by using proteasome inhibitors or an expression vector, *hes1* transcription is repressed by the persistently high Hes1 levels. On the other hand, treatment with cyclohexamide, an inhibitor of translation, or over-expression of a dominant-negative form of Hes1 (dnHes1), constitutively upregulates *hes1*

blocking its oscillations. Therefore, *hes1* oscillations requires both *de novo* synthesis and degradation of Hes1 protein and the negative feedback loop, in which Hes1 periodically represses its own transcription, is the central mechanism for the *hes1* oscillations both in cells and in the PSM (Hirata et al., 2002). Furthermore, using mathematical modeling, the authors predict that alterations in synthesis and degradation rates should change the oscillations period.

Similarly to *hes1*, *hes7* oscillations are also regulated by a negative feedback loop (Bessho et al., 2003). These authors characterized the expression of Hes7 in the PSM and identified three distinct phases of expression, similar to the ones observed for the cyclic genes. It has been shown by the same group that *lfng* mRNA oscillates in phase with *hes7* (Bessho et al., 2001a) and the authors further show that transcription of both *lfng* and *hes7* occurs only on regions negative for Hes7, suggesting that this protein represses the transcription of both genes making them cycle together (Bessho et al., 2003). In fact, chromatin immunoprecipitation (ChIP) analysis indicates that Hes7 interacts with both *hes7* and *lfng* promoters. Moreover, *hes7* and *lfng* transcription is constitutively up-regulated in the absence of Hes7 and down-regulated by protein stabilization, indicating that periodic repression by Hes7 protein establishes the cyclic transcription of both *hes7* and *lfng* (Bessho et al., 2003). Accordingly, in mice mutant for *hes7*, *lfng* expression is sustained, disrupting the oscillations (Bessho et al., 2001b). The half-life of the oscillating Hes7 protein is ~22min (Bessho et al., 2003). Increasing this half-life to 30min, by means of a point mutation, resulted in a halt of *hes7* oscillations and consequently disrupted somite formation (Hirata et al., 2004).

Regarding *lfng*, a negative feedback loop inducing periodic transcription repression has also been demonstrated to occur in chick (Dale et al., 2003). Misexpression of *lfng* by *in ovo* electroporation abolished cyclic gene expression of *lfng*, *hairy1* and *hairy2* in the chick PSM and perturbed the position of somite boundaries. The authors further show that *lfng* is a direct target of Notch in the chick, as had been previously shown for mouse (Cole et al., 2002; Morales et al., 2002) and that this regulation is made by the NICD because preventing its activation leads to a blockage of *lfng* oscillations. Thus, the authors identified a negative feedback loop acting in the chick PSM in which Lfng protein produced *via* a Notch-dependent mechanism inhibits Notch signalling and thus downregulates *lfng* expression (Fig. 1.3B). Due to the rapid turnover of Lfng protein its negative effect on Notch signalling will be transient and periodic (Dale et al., 2003).

Negative feedback loops between short-lived transcripts and proteins also appear to control the cyclic expression of *her1* and *her7* genes in the zebrafish embryo, similarly to what has been described for chick and mouse (Lewis et al., 2009).

## 1.11 WAVEFRONT OF DIFFERENTIATION

The second component postulated by the Clock-and-Wavefront model (Cooke and Zeeman, 1976), the wavefront of differentiation, was described a few years after the finding of the segmentation molecular clock. To analyse the commitment of PSM for segmentation, several A-P inversions of one-somite length were performed in the chick embryo and the correct A-P specification was evaluated by the expression of the Notch ligand *dll1*, present in the caudal portion of the somites (Dubrulle et al., 2001). When the orientation of S0 or S–I was inverted, the formed somite exhibited reversed polarity, indicating that this region is already committed with respect to its RC polarity segmentation. Inversion of more caudal PSM regions (S-V to S-XII) leads to a normal segmentation and polarity, suggesting that this tissue is still undetermined. However, when the inversion was performed at the level of somites –II to –IV, formation of ectopic boundaries and abnormal A-P segregation was observed, indicating that segmental determination takes place around somite –IV. Because this position is not fixed but regresses with the posterior elongation of the embryo, the authors designated it as determination front (Dubrulle et al., 2001). *Fgf8* mRNA, expressed in the caudal portion of the PSM (Crossley and Martin, 1995), seemed to be a good candidate to explain the segmental determination observed in the PSM. In fact, *fgf8* exhibits an A-P gradient of expression which seemed to coincide with the determination front level (Dubrulle et al., 2001). *In ovo* electroporation of *fgf8* strongly affected segmentation, as evidenced by the complete absence of somites or the formation of smaller ones, indicating that high Fgf levels maintain PSM cells in an undifferentiated state while lower levels are necessary for somite formation (Dubrulle et al., 2001). These authors also performed a series of experiments to change the axial level of the determination front. Grafting of a bead soaked in Fgf8 in the PSM leads to a rostral displacement of the determination front and generation of smaller somites since fewer cells integrate the forming somite. On the other hand, inhibiting the kinase activity of the Fgf receptors by culturing the embryo in the presence of SU5402, has the opposite effect: the determination front is shifted caudally and more cells are included in the forming somite, thus generating bigger somites (Dubrulle et al., 2001). In both cases, the phase of expression of *hairy2* and *lfng* genes was unaltered. The nature of the *fgf8* mRNA gradient was analysed by the same group (Dubrulle and Pourquie, 2004). Using an intronic RNA probe, *fgf8* nascent RNA was detected only in the tailbud region, both in mouse and chick and mature *fgf8* graded expression was detected throught the PSM even after incubation with the transcription inhibitor actinomycin D. Thus, the *fgf8* gradient observed in the caudal PSM is not due to active

transcription but rather to mRNA decay - the *fgf8* transcripts are produced in the tailbud and inherited by their descendants in the PSM and neural tube, thus generating a gradient (Dubrulle and Pourquie, 2004). The expression behaviour of *fgf8* mRNA and Fgf8 protein correlates with a gradient of activity of the Fgf signaling pathway (Dubrulle and Pourquie, 2004). On binding to Fgf receptor 1 (FgfR1), which is the only FgfR expressed in the PSM, Fgf can activate several intracellular effectors including the mitogen-activated protein kinase (MAPK), extracellular-signal-regulated kinase (ERK) and phosphatidylinositol-3-OH kinase (PI3K) (reviewed in Bottcher and Niehrs, 2005). In fact, Fgf8 PSM gradient correlates with a graded A-P expression of phosphorylated Akt, kinase acting downstream of PI3K in mouse (Dubrulle and Pourquie, 2004) and with a gradient of activated ERK in chick and zebrafish (Sawada et al., 2001; Delfini et al., 2005). The Fgf/ERK signaling is in fact regulating cell motility along the A-P axis and constitutive activation of the MAPK/ERK pathway in the chick produced the same results as over-expressing *fgf8* – PSM cells retained a mesenchymal nature and did not incorporate epithelial somites (Delfini et al., 2005). Culturing the embryos in the presence of SU5402 to block Fgf signaling or U0126 to prevent ERK activation did not affect *lfng* cyclic expression, suggesting that Fgf/ERK activity is not required to produce oscillations (Delfini et al., 2005).

Evidence for a gradient of Wnt/β-catenin signaling along the PSM has also been provided. Binding of the Wnt ligand (e.g. Wnt3a) to the Frizzled membrane receptor ultimately leads to the release of β-catenin from a destruction complex, which can then translocate to the nucleus where it binds Tcf/Lef transcription factors to modulate target genes. In the absence of the ligand, cytoplasmic β-catenin is recruited to a protein complex assembling around Axin where it is target for degradation via the proteosome (reviewed in Hayward et al., 2008). *Axin2* expression, an inhibitor of the Wnt pathway, is directly regulated by the Wnt/β-catenin signaling and presents an oscillatory graded expression along the PSM peaking in the tail bud (Aulehla et al., 2003). Constitutive expression of *axin2* in the PSM of transgenic mice resulted in the formation of larger somites. Moreover, increasing Wnt3a concentration using beads covered in *wnt3a*-expressing cells induced the formation of smaller somites indicating that, as Fgf8, Wnt3a has an important role in defining the segmented barrier position (Aulehla et al., 2003). In fact, Wnt/β-catenin signaling has been further implicated in PSM differentiation and determination front positioning. β-catenin protein, a key intracellular component of the pathway, is expressed in the mouse PSM as a clear A-P citoplasmatic-to-nuclear gradient – it predominantly localizes to the nucleus in the posterior PSM while is mainly cytoplasmic in the anterior PSM (Aulehla et al., 2008). The conditional gain-of-function of *β-catenin* using the *T-Cre* system disrupted the graded expression of this gene

inducing an elevated β-catenin nuclear staining along the entire PSM (Aulehla et al., 2008). In these mutants, a Wnt and Fgf gain-of-function was observed resulting in an extended immature PSM with no somites being formed (Aulehla et al., 2008; Dunty et al., 2008). Part of the phenotype observed after β-catenin stabilization is mediated indirectly by Fgf signaling, indicating that these pathways synergize to maintain the posterior PSM in an immature state (Aulehla et al., 2008). Oscillation of the segmentation clock genes continued normally in the mutants but no regression of the oscillatory domain in the anterior PSM was observed, indicating that cyclic gene expression is not achieved by variations in β-catenin nuclear levels but that a decrease in the protein's nuclear levels is required for the cessation of the oscillations in the anterior PSM and formation of morphological somites (Aulehla et al., 2008).

The position of the determination front is further refined by a gradient of retinoic acid (RA), the major active derivative of vitamin A (retinol), antagonizing the Fgf gradient (Diez del Corral et al., 2003). Although RA cannot be visualized directly, the retinaldehyde dehydrogenase enzyme *raldh2*, required for RA synthesis is strongly expressed in the somites and in the rostral PSM of the chick embryo while retinoic acid receptors (RARα,β,γ and RXRα,γ) are expressed in the neural tube. Treatment with the RA agonist TTNPB induces a drastic reduction or loss of *fgf8* levels while absence of RA signaling in the vitamin A deficient (VAD) quail embryos leads to an increase in *fgf8* domain and consequent formation of smaller somites. Moreover, when Fgf8-soaked beads are placed in contact with the anterior PSM, *raldh2* expression is repressed. These results indicate that there is mutual inhibition of the RA and Fgf signaling pathways, which are involved in defining the somite boundary formation region and in the regulation of PSM differentiation (Diez del Corral et al., 2003).

The opposition of Fgf/Wnt3a and RA in the PSM defines the position of the future somite boundary during the process of segmentation and thus the transition from FGF to RA signalling constitutes a differentiation switch in the extending body axis. High Fgf levels in the posterior PSM maintain this tissue under an undifferentiated state and it is therefore designated as undetermined PSM; the tissue located above this front (localized around S-IV) is called determined PSM and corresponds to three to four already determined somites (Dubrulle et al., 2001) (Fig. 1.4). Wnt signaling has been also implicated in the regulation of this transition (Olivera-Martinez and Storey, 2007). Fgf signaling in the caudal chick embryo region induces expression of *wnt8c*. As Fgf levels decrease around the determination front region, *wnt8c* persists and is responsible for inducing *raldh2* expression and thus RA activity, which will in turn further inhibit *fgf8* and *wnt8c* (Olivera-Martinez and Storey, 2007). Interestingly, a similar relationship

between these pathways has been observed in limb proximal-distal development where Fgf and Wnt pathways promote distal outgrowth while RA has an opposing proximalizing role (reviewed in Tabin and Wolpert, 2007). This is probably a fundamental and conserved molecular mechanism that regulates differentiation progression during the development of segmented structures.

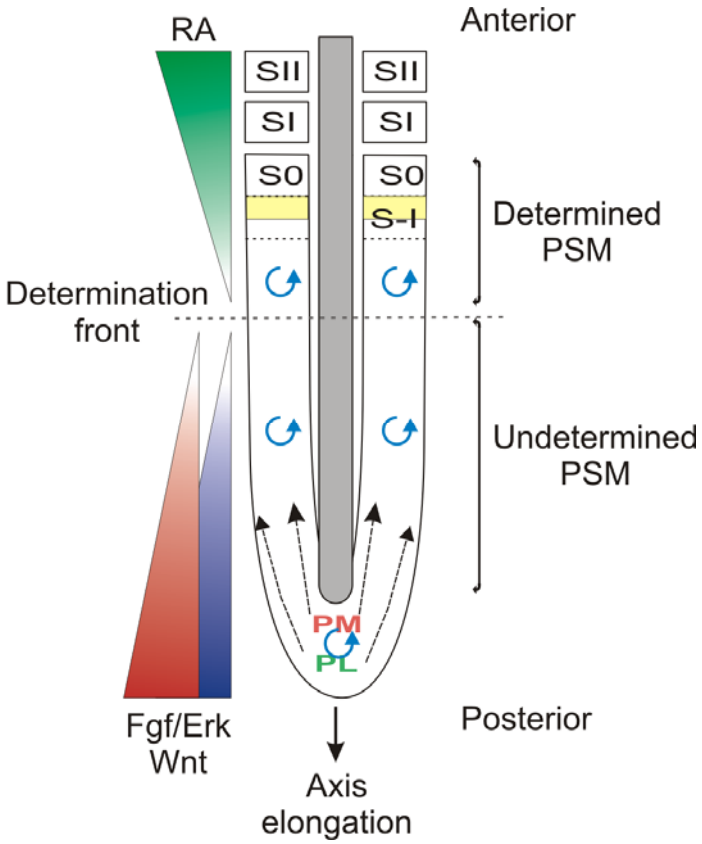


**Figure 1.4. Signaling activity during somitogenesis.** Schematic representation of the integration of distinct signaling activities in the PSM. Molecular clock oscillations (light blue spiral symbol) are observed in the somitic precursor cells in the tailbud region and along the entire PSM. The system of opposing gradients of Fgf/Erk (red triangle), Wnt (purple triangle) and Retinoic Acid (RA, green triangle) positions the determination front (dashed line) in the PSM (Dubrulle et al., 2001; Aulehla et al., 2003; Diez del Corral et al., 2003; Dubrulle and Pourquie, 2004). High Fgf/Wnt levels maintain the posterior PSM cells in an undifferentiated state and thus this region is designated undetermined PSM; the PSM tissue above the determination front is considered to be determined and containing three-four presumptive somites (Dubrulle et al., 2001). The determination front marks the position where the next prospective boundary will form, thus shaping somite size. Confrontation between the molecular clock oscillations and the determination front is considered to define segment formation by inducing *mesp2* in the anterior PSM (light yellow) (Morimoto et al., 2005; Yasuhiko et al., 2006). During somitogenesis, the embryo continuously grows and elongates posteriorly. S0 represents the forming somite, SI and SII the two most recent formed somites. PM and PL represents prospective medial and lateral PSM, respectively; arrows indicate future cell position in the PSM.

## 1.12 WNT- AND FGF- REGULATED CYCLIC GENES

In addition to the Notch pathway, many components of the Fgf and Wnt signalling have been shown to oscillate in the mouse PSM, as revealed by an exhaustive microarray analysis (Dequéant et al., 2006) (Summarized in Table 1.1). Notch and Fgf pathway genes oscillate in phase with each other while genes belonging to the Wnt signaling pathway are out-of-phase with Notch-Fgf. This had been previously shown in the mouse for the only Wnt component known to cycle thus far – *axin2*, an inhibitor of the Wnt pathway (Aulehla et al., 2003). Each of the three pathways plays multiple roles in the PSM and the molecular interactions between them have been analysed (Summarized in Table 1.2).

Oscillating mouse genes belonging to the Fgf pathway include the negative regulators *sprouty2* and *4* (*spry2* and *4*), *dusp4*, *dusp6* (also known as *mkp3*) and *snail1* (Dale et al., 2006; Dequéant et al., 2006; Niwa et al., 2007; Hayashi et al., 2009). Regarding the chick embryo, to date only *snail2* transcriptional repressor has been shown to present an oscillatory behaviour (Dale et al., 2006). The fact that Notch and Fgf genes oscillate in synchrony suggest that they might regulate each other. However, the data obtained so far is contradictory. In *hes7* null mouse, oscillatory expression of Fgf targets *dusp6*, *spry4* and *snail1* is still observed, which is thought to account for the formation of irregular somites in those mutants (Ferjentsik et al., 2009). Moreover, expression of *snail* genes in mouse and chick does not depend on Notch signaling (Dale et al., 2006). Dynamic *spry2*, *dusp6* and *snail1* expression are not affected in mouse embryos null for *rbpjk*, which abolish Notch signalling (Dequéant et al., 2006; Ferjentsik et al., 2009). However, null mutants for *rbpjk* still present weak NCID activity, which could explain the observed results. In fact, when Notch signaling is completely abolished in *psen1*/*psen2* double mutants, the expression of NCID, *snail1*, *dusp6* and *spry2* is no longer detected in the PSM (Ferjentsik et al., 2009), suggesting that Notch signaling regulates Fgf cyclic expression. Contrastingly, conditional deletion of the Fgf receptor 1 (Fgfr1) in mouse, blocks the oscillation of *lfng* and *hes7* genes (Niwa et al., 2007; Wahl et al., 2007). It has also been shown that Fgf stimulation in cultured cells induces cycles of both *hes1* mRNA and protein with a two hour periodicity (Nakayama et al., 2008). Similarly, in zebrafish Fgf induces *her13.2* expression, which is required for *her1*, *her7* and *deltaC* oscillations (Kawamura et al., 2005). Pharmacological treatments have shown that oscillations of Notch genes both in mouse and chick do not require Fgf signaling (Delfini et al., 2005; Gibb et al., 2009). The analysis performed thus far has not yet allowed unravelling of the relationship between Fgf and Notch pathways and therefore further studies are required to clarify

this question. There is a report however indicating that these two pathways probably work together: generation of mouse *hes7* oscillations in the posterior PSM requires Fgf signaling and are maintained anteriorly by Notch (Niwa et al., 2007).

Genes from the Wnt cluster oscillating in opposite phase of Notch-Fgf genes include the negative regulators *axin2* (Aulehla et al., 2003), *dkk1*, *dact1* and *c-myc* (Dequéant et al., 2006). Recently it was demonstrated that chick homologues for these Wnt regulators (*dac1*, *axin2* and *nkd1*) do not present a dynamic expression (Gibb et al., 2009), which does not exclude the possibility that other Wnt genes may cycle in the chick. Several experimental evidences have suggested that Wnt signaling is upstream of Notch, namely the analysis of the *wnt3a* hypomorphic mutant *vestigial tail* (*vt*) embryo, in which *wnt3a* expression in the tail bud region is greatly reduced (Greco et al., 1996). In these embryos, *dll1* and *notch1* expression is downregulated in the tail bud and *lfng* oscillatory behaviour is disrupted, indicating that Wnt regulates Notch signaling and this is probably mediated by *axin2* (Aulehla et al., 2003). Furthermore, mice carrying a truncated form of Lef1 that can interact with β-catenin but fails to bind DNA, present a decreased expression of *dll1*, *notch* and *lfng* (Galceran et al., 2004). In fact, it has been shown that *dll1* and *notch1* promoters posses multiple potential binding sites for Lef1/Tcf transcription factors and *dll1* presents also a Tbx6 binding site, suggesting that they are directly regulated by the Wnt pathway (Galceran et al., 2004; Hofmann et al., 2004). Contrastingly, *axin2* oscillations are lost when Notch signaling is completely abrogated as in *psen1*/*psen2* double mutants but are not affected in *hes7* or *rbpjk* mutants (Ferjentsik et al., 2009). Pharmacological inhibition in both chick and mouse of either Notch (using DAPT) or Wnt (using CKI-7, a casein kinase inhibitor) suggests the existence of a crosstalk between the two pathways since treatment induces the downregulation of target genes from the reciprocal pathway (Gibb et al., 2009). An important question is at what level of the signalling transcription pathway this crosstalk occurs. The description of oscillatory behaviour for *nkd1* and *nrarp* genes, downstream of the Notch signaling and regulators of Wnt pathway (Dequéant et al., 2006) suggest a possible cross-regulation between these two pathways. Pharmacological experiments show that *nrarp* dynamic expression is Notch-dependent in chick, mouse and zebrafish (*nrarp-a*) embryos but Wnt dependency has only been observed in mouse (Wright et al., 2009). Gain or loss of *nrarp* function does not interfere with Notch-related cycling genes (Wright et al., 2009). In mouse, initiation of *nrarp* expression within the PSM seems to require Wnt and later in development is maintained by *lfng* (Sewell et al., 2009). A similar regulation has been observed for *nkd1* in mouse, which seems to require *wnt3a* for transcription and *hes7* for the oscillatory behaviour (Ishikawa et al., 2004). Although the exact role of these

genes in the crosstalk regulation between Notch and Wnt has not yet been clarified, they seem to constitute a promising link between the pathways and may play a pivotal role in the regulation of the somitogenesis clock.

**Table 1.2** – Mutations in key genes belonging to the Notch, Wnt and Fgf signaling pathways and subsequent phenotype observed in the expression of genes either involved in the molecular clock or in the wavefront of differentiation. Only mutations bringing insight into the putative cross-talk between pathways were considered for analysis.

| Pathway | Mutated gene | Effects in gene expression | References |
|---|---|---|---|
| **Notch** | *dll1* null | ↻ *axin2*<br>✓ *fgf8*, *wnt3a* | (Aulehla et al., 2003) |
| | *hes7* null | ↻ *axin2, snail1, dusp6, spry1*<br>∅ *lfng, nrarp, dusp4, nkd1* | (Ferjentsik et al., 2009)<br>(Niwa et al., 2007)<br>(Ishikawa et al., 2004) |
| | *notch* over-expression | ↻ *snail2* | (Dale et al., 2006) |
| | *psen1*/*psen2* null | ∅ *axin2*, *snail1*, *dusp6*, *spry2* | (Ferjentsik et al., 2009) |
| | *Rbpjk* null | ↻ *spry2, dusp6, axin2, hes7, snail1*<br>∅ *lfng* | (Dequéant et al., 2006)<br>(Ferjentsik et al., 2009)<br>(Dale et al., 2006) |
| **Wnt** | sustained *axin2* | ∅ *lfng* | (Aulehla et al., 2003) |
| | *β-catenin* Gof | ↑ *dusp6, spry2* | (Dunty et al., 2008) |
| | truncated *lef* | ∅ *lfng*<br>↓ *dll1, notch* | (Galceran et al., 2004) |
| | *wnt3a* mutant vt | ∅ *snail1*, *lfng, axin2, nkd1*<br>↓ *fgf8, dll1, notch* | (Dale et al., 2006)<br>(Aulehla et al., 2003)<br>(Ishikawa et al., 2004) |
| **Fgf** | conditional *fgfr1* | ∅ *lfng*, *hes7, spry2, axin2, snail1* | (Wahl et al., 2007)<br>(Niwa et al., 2007) |
| | *snail2* over-expression | ∅ *lfng* | (Dale et al., 2006) |

↻ normal oscillations; ✓ normal expression; ∅ disrupted oscillations; ↑ increased expression; ↓ decreased expression

Regarding the interplay between Fgf and Wnt, analysis of the *wnt3a vt*/*vt* mutants shows that *wnt3a* regulates *fgf8* expression in the posterior PSM (Aulehla et al., 2003) and *snail1* cyclic expression (Dale et al., 2006). Moreover, β-catenin gain of function causes an upregulation of *dusp6* and *spry2* expression (Dunty et al., 2008). Opposingly, *axin2* expression is abnormal in mouse presenting a conditional deletion of Fgfr1 (Wahl et al., 2007). This indicates that Fgf and Wnt also co-regulate each other in the PSM.

Several studies have tried to establish a hierarchy between the three signaling mechanisms operating in somitogenesis regulation (Summarized in Table 1.2). However, it has not been easy to exactly pinpoint a regulation chain or to identify putative molecules making the bridge between

the pathways. This only shows that due to the developmental importance of somite formation, this process is extremely well regulated. In order to ensure the robustness of somitogenesis, these signalling cascades are probably working synergistically, creating a complex and highly efficient signaling network.

## 1.13 CLOCK AND WAVEFRONT INTERACTION

It is generally accepted that the confrontation between the molecular gradients defining the determination front and the molecular clock oscillations specifies segment formation in the anterior PSM (Fig. 1.4). What is not clear however is how this is achieved. Aulehla et al. (2003) proposed that Wnt3a graded signaling in the mouse PSM activates Wnt cyclic expression, which in turn regulates Notch gene oscillation (Aulehla et al., 2003). Moreover, a decrease in β-catenin protein's nuclear levels in the anterior PSM seems to be required for the cessation of the oscillations and formation of morphological somites (Aulehla et al., 2008). In fact, in β-catenin gain-of-function mutants, the number of *lfng* stripes in the PSM increased significantly corresponding to multiple oscillations that sweep the PSM but do not regress anteriorly because no somites are formed due to the increased β-catenin nuclear levels. These results suggest that Wnt high levels in the posterior PSM provides a permissive environment for cyclic expression and that the arrest of the clock oscillations in the anterior region requires the downregulation of Wnt signaling.

Once cells reach the anterior PSM, the oscillatory activity and graded expression need to be converted into a cell fate change, in which somite-forming units are specified (Fig. 1.4). This is accompanied by several changes at the molecular level, being one of them the periodic activation of the expression of *mesp2* in the anterior PSM, an essential factor that is activated to define the future somite boundary position (Saga et al., 1997; Morimoto et al., 2005). It has been shown that Notch oscillations are arrested in the anterior PSM by *mesp2* and boundaries are formed between Notch- activated and inactivated domains (Morimoto et al., 2005). *Mesp2* is itself activated by Notch: *tbx6* expressed along the entire PSM directly binds *mesp2* promoter while high NCID levels in the anterior PSM strongly enhances *mesp2* activation by *tbx6* (Yasuhiko et al., 2006). High levels of Fgf and Wnt signalling in the posterior PSM repress the expression of *mesp2* in this region (Delfini et al., 2005; Dunty et al., 2008), dictating that the gene activation occurs only when Fgf and Wnt levels drop below a determined threshold.

## 1.14 CLOCK REGULATION MECHANISMS

Considering all data presented here, one can say that embryo segmentation is a precisely regulated mechanism that requires timely gene expression oscillations. In fact, this has been theoretical predicted and experimentally observed. When the expression of a given gene or protein is either knocked down or constitutively activated, phenotypic defects are depicted, indicating that the consecutive transitions between maximal and minimal molecular values observed during oscillations are necessary for proper development. In fact, over- or sustained expression of a given gene induces segmentation defects and consequently severe skeletal malformations such as disorganized and fused vertebrae and ribs. For example, mutant mice for *lfng* or *hes7* present short tail and trunks, the vertebrae formed are shorter in length and the ribs are usually fused and bifurcated (Evrard et al., 1998; Zhang and Gridley, 1998; Bessho et al., 2001b). Moreover, sustained expression of *lfng* disrupts molecular oscillations and normal somitogenesis, creating offspring with shorter and kinked tails and fused vertebrae and ribs (Bessho et al., 2001b; Serth et al., 2003). Noticeably, this oscillatory behavior also seems to be implicated in stem cell fate determination: cyclic *hes1* expression in embryonic stem cells seems to contribute to the heterogeneous response of the cells and low or high *hes1* levels are determinant to specify cells to either a neuronal or mesodermal fate, respectively (Kobayashi et al., 2009).

These synchronous oscillations have intrigued developmental biologists since they were first described (Palmeirim et al., 1997; Skipper, 2004) and several attempts have been made to interfere with this robustly regulated mechanism. The description of several Notch-independent genes exhibiting an oscillatory pattern in the PSM (Dequéant et al., 2006) has brought new insight into the molecular orchestration of the oscillations. In an effort to better understand this oscillatory behaviour, several attempts have been made in order to alter the clock periodicity. In fact, Mathematical modeling predicts that alterations in protein synthesis and degradation rates should mchange the oscillations period (Hirata et al., 2002; Momiji and Monk, 2008). Recently, an increase in the somitogenesis period was experimentally achieved. Incubating chick embryo explants in the presence of the Wnt inhibitor CKI-7 for 4 hours lead to the formation of one less somite boundary than the control and slowed down the pace of the clock to 115-120 minutes, representing the first time that the periodicity of the clock was experimentally altered (Gibb et al., 2009). The authors were however unable to accelerate the pace of the clock even after over-activating the Wnt signaling pathway. Disruption of the Dll-Notch coupling in zebrafish also induced an increase in the somitogenesis period and somite size until the complete lost of

synchrony, when no more somites were formed (Herrgen et al., 2010). Exactly how these delays in the clock are achieved molecularly is still unclear.

Several aspects of the molecular clock regulation mechanisms remain unanswered, especially regarding the level of crosstalk between the different pathways regulating the oscillatory behaviour. Presently, it is impossible to determine which genes are driving oscillations or permitting them. Furthermore, it is thought that the Notch, Wnt and Fgf oscillators need to be somehow entrained by a so-called pacemaker to ensure that oscillations occur with the correct periodicity. The nature of this pacemaker overseeing the molecular clock oscillations and how this control is made has not yet been clarified. The past years have provided important progress in the understanding of vertebrate embryo segmentation. A big step forward was taken with the establishment of the real time bioluminescence imaging technique in mouse embryos, which constitutes a powerful tool to further study the mechanics of clock oscillation and regulation *in vivo* (Masamizu et al., 2006). However, the knowledge and understanding of the molecular events underlying it are still limited.

## 1.15 VERTEBRAL SEGMENTATION DISORDERS IN HUMAN

A better comprehension of the molecular mechanisms underlying somite formation is required not only for the sake of basic developmental biology but also with the aim of dissecting the origin of human skeletal malformations. In fact, in the last years several mice mutant models have been produced to analyze the function of the somitogenesis genes and these have proven to constitute good tools to understand congenital vertebral malformations in human. In the cases when the generated mutation is not lethal and it is possible to follow the development of the embryos, the most common defects observed are shorter trunks with fused or bifurcated vertebrae and ribs (reviewed in Andrade et al., 2007). The same segmentation problems are observed for example in human spondylocostal dysostosis (SD), in which patients exhibit a short trunk due to multiple hemi-vertebrae formation accompanied by rib fusions, bifurcations and deletions (reviewed in Turnpenny et al., 2007; Giampietro et al., 2009). Mutations in Notch-regulated genes such as *dll3*, *lfng* and *mesp2* have been found to induce spondylocostal dysostosis. Alagille syndrome is a disorder characterized by developmental abnormalities of the liver, heart, eye and skeleton, which has been associated with mutations in *jagged1*, a Notch ligand, and *notch2* genes. Mutations of *fgfr1-3* in human have also been shown to result in skeletal disorders, including fusion of the craniofacial sutures and short-limbed dwarfisms (reviewed in Chen and Deng, 2005).

In human, it is considered that somitogenesis takes place between 20 and 35 days after conception and formation of each pair of somites takes around 4-6 hours (reviewed in Turnpenny et al., 2007). Although a human segmentation molecular clock has not yet been demonstrated experimentally due to the inability to examine early developmental events in human embryos, the above described phenotypes are very similar to the mutation phenotypes observed in the mice models. Promising results were obtained by performing microarray analysis in a human mesenchymal stem cell population derived from umbilical cord blood (William et al., 2007). Quantitative PCR analysis confirmed that *hes1* gene oscillates in these cells with a 5h periodicity. Hence, the data obtained so far strongly suggests that a oscillatory mechanism underlying axial skeleton formation may be conserved in humans.

## 2. HEDGEHOG SIGNALING

Members of the Hedgehog (Hh) family of signaling molecules have been shown to play pivotal roles in many processes of embryonic development. The activity of this family of genes is necessary for fundamental embryonic events that include growth, patterning and morphogenesis of most tissues and structures within the vertebrate body. Such a variety of functions is possible due to the complex regulatory network of the pathway and because cellular response to Hh is not a straightforward mechanism and depends on the dose of signal received, the type of responding cell and the time period during which cells are exposed to the signal (reviewed in Ribes and Briscoe, 2009).

The *hh* gene was first identified in *Drosophila melanogaster* in a large screen for mutations that impaired the normal development of the larva's body plan. In vertebrates, three distinct *hh* genes have been identified both in avian and mammalian embryos: *desert hedgehog* (*dhh*), *indian hedgehog* (*ihh*) and *sonic hedgehog* (*shh*) (reviewed in Varjosalo and Taipale, 2008). Hh proteins seem to have distinct functions in patterning the embryonic body but in some cases their function can be partly redundant (reviewed in Ingham and McMahon, 2001; Varjosalo and Taipale, 2008). *Dhh* is mainly expressed in the gonads and null mutants for the gene do not present an important phenotype but the males are infertile due to the absence of mature sperm. Contrastingly, 50% of the *ihh* mutant embryos are lethal due to a failure in the yolk-sac vasculature development whereas surviving embryos present several bone defects. *Shh* has been implicated in the regulation of several events during early embryonic development. It is the *hh* gene with the broader expression pattern and during organogenesis is implicated in the development of most epithelial tissues. It can

be detected in the embryo midline structures such as the notochord (no) and the floor plate of the neural tube (fp), where it is implicated in the patterning of the neural tube and in the establishment of the left-right and dorso-ventral body axes. *Shh* is also expressed in the zone of polarizing activity (ZPA), a major signaling centre of the growing limb bud, where it is critical for the patterning of the distal elements of the limb. Deletion of *shh* results in diverse severe phenotypes including cyclopia and defects in ventral tube and somite patterning, which later in development leads to distal limb malformations and absence of vertebrae and most of the ribs (Chiang et al., 1996; reviewed in Varjosalo and Taipale, 2008). Due to the importance of Shh in embryo development and its implication in different embryologic events, the next sections will mainly focus on this member of the Hh family.

## 2.1 FORMATION OF THE HEDGEHOG GRADIENT

Hh proteins have been shown to operate as morphogens, small diffusible molecules able to travel along a considerable distance establishing a gradient of activity. The notion of morphogen was firstly described in 1969 by Wolpert (Wolpert, 1969; reviewed in Dessaud et al., 2008). In this work, Wolpert proposed the "French Flag" model, in which a secreted substance emanating from a given source spreads through the tissue to establish a gradient of activity. Around the source, the signal concentration is high but it will decrease over the traveled distance until no signal is detected. According to its concentration, this signal patterns the tissue into three domains with different gene activity, which corresponds to the striped colors of the French flag. Thus, a morphogen presents two important characteristics: it is able to act on cells at a distance from the source (which constitutes the morphogen's signaling range) and secondly, it is capable of inducing distinct gene expression based on its concentration. Both criteria are met by Shh in the limb bud and in the neural tube. In the limb bud, Shh has been shown to act over ~300µm (Lewis et al., 2001) and specification of posterior and anterior digits is induced by high and low Shh concentration, respectively (Riddle et al., 1993; Yang et al., 1997; Lewis et al., 2001). Regarding the neural tube, it has been shown that different concentrations of recombinant Shh protein induce distinct gene expression profiles along the ventral neural tube, responsible for subsequent specific neuronal induction (Ericson et al., 1997; Wijgerde et al., 2002). Moreover, in this structure, Shh has been shown to travel over 20 cell diameter (~200 µm) (Briscoe et al., 2001). However, the French flag model has been challenged over the years and some of its assumptions seem not to apply to Shh signaling. For example, in the model, the responding cell is more or less passive to

the signal, which does not seem to happen in the case of Shh. It is now generally accepted that a molecular interaction between the signaling molecule and the responding cell occurs and that this dialogue is crucial for the correct establishment of the gradient (reviewed in Dessaud et al., 2008; Ribes and Briscoe, 2009). In fact, the receiving cell's response to the ligand influences the gradient range of activity and, over time, cells become desensitize to the signaling molecule. Thus, the action of a morphogen seems to be a dynamic process, as will be further discussed in the following sections.

## 2.2 HEDGEHOG PROCESSING AND RELEASE

In order to became fully active and be released from the producing cell, Hh proteins undergo a series of post-translational modifications (reviewed in Ingham and McMahon, 2001; Varjosalo and Taipale, 2008). First, the protein is auto-catalytically cleaved generating a C-terminal domain without signaling activity and an N-terminal domain that possess signaling functions (HhN) and presents a cholesterol group at its C-terminus, which is responsible for its association with the plasma membrane. Palmitic acid is then added to its N-terminus by the acetiltransferase *skinny hedgehog* (*skn*). Thus, biological active Hh (HhNp, in which p indicates processed) is cholesterol modified at its C-terminus and palmitoylated at the N-terminus and both lipid modifications are essential for the secretion and activity of the protein (Lewis et al., 2001; Chen et al., 2004) (Fig. 2.1A). In fact, mice in which ShhN protein lacked the cholesterol modification formed posterior digits but not the anterior ones, indicating that the short-ranged signaling required for posterior digit formation was unaffected but the long-range signaling was insufficient to generate anterior digits (Lewis et al., 2001). A similar defective long-range Shh signaling was observed in mice with ShhN lacking the palmitoylation modification: anterior digit formation was disrupted and the neural tube was not correctly patterned along its A-P axis (Chen et al., 2004). This was also noticed in cells tranfected with Shh mutants lacking the N-terminal palmitic acid, the cholesterol moiety or both (Etheridge et al., 2010). ShhNp could be detected a few cell diameters away from the producing cell. However, Shh lacking the palmitic acid was still spread in a gradient but presented an increased range of distribution. Contrastingly, in the absence of the cholesterol modification Shh was scarce in the extracellular space and no obvious gradient was observed whereas in a mutant lacking both modifications was undetectable. These results show that the lipid modifications present distinct functions: the cholesterol anchor is required for Shh retention in the extracellular matrix, whereas the palmitoylation sharpens the Shh gradient (Etheridge et al., 2010).

Long-range Shh signaling has been shown to require Dispatched 1 (Disp1) a transmembrane, sterol-sensing domain (SSD)-containing protein (Kawakami et al., 2002; Ma et al., 2002). Analysis of *disp1*$^{-/-}$ embryos shows that this protein is not required for Hh processing or synthesis as both Shh unprocessed and processed forms are detected in the mutant. However, ShhNp export in the absence of *disp* is defective (Ma et al., 2002; Etheridge et al., 2010). In fact, examination of protein distribution indicates that in wild type embryos, Shh is strongly detected in the notochord and extends outwards in a graded manner while in the mutant embryos it is detected only in the synthesis site, the notochord (Kawakami et al., 2002). This indicates that Disp1 in necessary for protein transport out of the producing cell and thus for the establishment of a graded activity and explains why *disp1* null mice present defective long- and short- ranged Shh signaling. It has been shown that Disp1 releases only the cholesterol modified protein and Shh lacking this lipid modification undergoes a Disp1-independent secretion (Tian et al., 2005; Etheridge et al., 2010). This observation can explain the defective long-range signaling observed when Shh protein lacks the cholesterol modification. This form does not require Disp1 for its release and thus is able to spread more rapidly through tissues, leading to a rapid diffusion of the ligand away from the source, which results in less protein reaching the target cells and thus a proper graded signal is not chieved, as predicted by computational modeling (Saha and Schaffer, 2006).

Analysis of the culture media of cells transfected with constructs encoding either ShhNp or Shh lacking cholesterol, palmitoylation or both modifications indicated that ShhNp associates with lipid raft domains in the membrane and is predominantly present in a multimeric complex, although some monomeric forms were also detected (Chen et al., 2004; Goetz et al., 2006). In fact, the multimeric forms are more bioactive than the monomeric ones, both *in vitro* and *in vivo* (Chen et al., 2004). Furthermore, cells lacking one or both lipid modifications were only able to form ShhN monomers, indicating that both modifications are essential for the formation of multimeric protein complexes (Chen et al., 2004; Goetz et al., 2006). Disp1 probably functions to facilitate the assembly and release of these complexes. However, the mechanism by which multimeric ShhNp is transported across the morphogenetic field over long distances is not clear yet. In *Drosophila* wing discs, several evidences point to a role of the heparin sulfate proteoglycans (HSPGs), components of the extracellular matrix, in Shh transport. In fact, it has been shown that ShhNp binds HSPGs through a highly conserved heparin-binding domain, the Cardin-Weintraub consensus sequence (Rubin et al., 2002). Moreover, in the absence of HSPGs the Hh signaling range is greatly reduced (Bornemann et al., 2004; Han et al., 2004). In the *Drosophila* wing, a class of HSPGs, the glypicans, has been implicated in the movement of Hh proteins across a field of cells (Han et al.,

2004). The authors propose that Hh is released by the producing cells through Disp1 and immediately captured by the glypicans on the cell surface. Hh is then displaced from one HSPGs chain to another towards more distant receiving cells. In fact, it has been shown that Hh oligomerization is essential for the interaction with HSPGs, required for the Hh long-range signaling (Vyas et al., 2008). HSPGs are present on the cell surface and on lipoproteins particles and mediate Hh transport either directly from cell to cell or in lipoproteins particles (see Fig. 2.1A). Indeed, mutants lacking the Cardin-Weintraub sequence do not present visible Hh clusters at the cell surface and fail to participate in paracrine signaling although signaling to adjacent cells occurs (Vyas et al., 2008). Thus, HSPGs seem to play an important role in *Drosophila* Hh long-range signaling; their role in vertebrate Hh signaling has not been clarified yet.

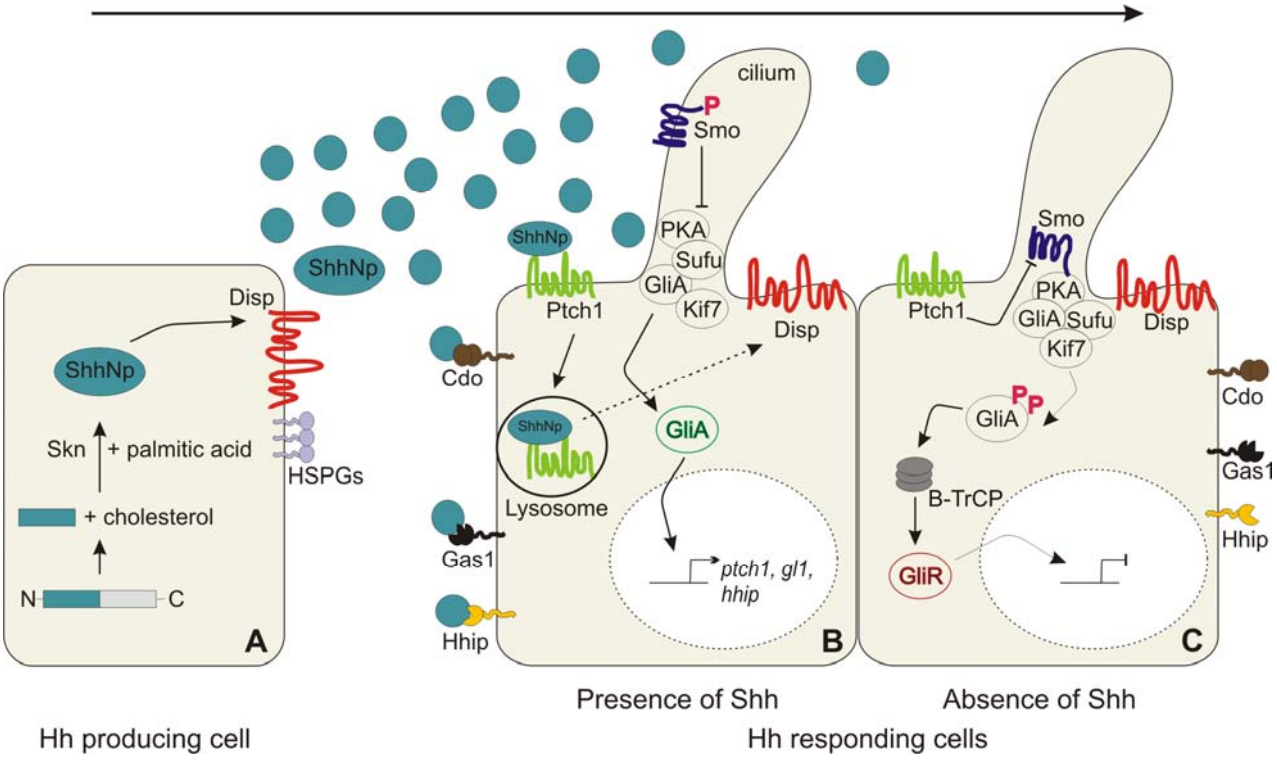


**Figure 2.1. Hedgehog signaling pathway. (A)** Production of an active Hedgehog protein involves firstly a protein auto-catalytic cleavage. The functional N-terminal domain is subsequently modified by the addition of a cholesterol group and a palmitic acid by Skinny Hedgehog (Skn) to yield a dually lipidated signaling protein (HhNp). Here represented is Sonic hedgehog (ShhNp) protein. ShhNp is then trafficked to the cell surface where it interacts with heparin sulfate proteoglycans (HSPGs) and is released from cells in a process mediated by the transmembrane protein Dispatched (Disp) (Kawakami et al., 2002; Ma et al., 2002), which localizes at the basolateral side of the cells (Etheridge et al., 2010). Shh proteins can then move from cell to cell in association with HSPGs or can be transported by lipoproteins particles (Vyas et al., 2008). Arrow on the top indicates direction of the decreasing gradient. **(B)** On

responding cells, the binding of Shh to Patched1 (Ptch1) receptor releases the repression of the transmembranar receptor Smoothened (Smo), allowing its translocation to the cell's cilium, which is thought to be mediated by the kinesin motor protein Kif7 (Liem et al., 2009). Ptch1-Shh are internalized and degraded in lysosomes (Incardona et al., 2000). Internalized Shh can also be reloaded for re-secretion (dotted arrow) (Etheridge et al., 2010). Phosphorylated Smo inhibits the proteolytic processing of the Gli transcription factors and activated Gli proteins (GliA) are released from a complex containing the Gli protein, Kif7, protein kinase A (PKA) and the kinase supressor of fused (Sufu). GliA is then translocated into the nucleus to activate target gene expression. At the cell surface, Shh also interacts with the Hedgehog binding protein (Hhip1), Cdo, Bco and Gas1 proteins, thus regulating the range and level of Hh signaling. **(C)** In the absence of Shh, Ptch1 represses the activity of Smo and its translocation to the primary cilium. Gli proteins are therefore phosphorylated by PKA and Sufu and thus targeted for proteasome (β-TrCP) processing. The processed forms of Gli present repressor activity (GliR), inhibiting the transcription of Shh targets in the nucleus. For a review, see Varjosalo and Taipale, 2008.

## 2.3 HEDGEHOG SIGNAL TRANSDUCTION

Identification of the vertebrate components of the Hh intracellular signaling has shown that many of the key signaling molecules of the pathway are conserved in vertebrates. However, the signaling mechanisms are not completely understood and present significant differences compared to *Drosophila.* Hh signal in the receiving cells is transduced through Hh binding to Patched (Ptch), a multipass transmembrane protein containing a SSD domain, similarly to Disp. Both mice and chick posses two Ptch isoforms, Ptch1 and Ptch2 (Goodrich et al., 1997; Pearse et al., 2001), which are able to recognize all Hh ligands and to associate with another Hh transmembranar receptor, Smoothened (Smo) (Carpenter et al., 1998). However, mice Ptch2 lacks a C-terminal tail, which has been proposed to affect its ability to inhibit Smo activity and to be responsible for signaling differences between Ptch1 and Ptch2 (Carpenter et al., 1998). Ptch1 is expressed broadly in mice embryos whereas high Ptch2 levels are only detected in specific tissues, such as the skin and testis, suggesting that Ptch1 may be the primary mediator of the Shh signaling (see (Carpenter et al., 1998) and references therein). In fact, mice deficient in *ptch2* are defective but viable while loss of *ptch1* leads to the complete inactivation of the Hh pathway, the neural tube fails to close and embryos die around embryonic day (E) 9.0 and 10.5 (reviewed in Varjosalo and Taipale, 2008). Contrastingly, in the chick embryo, Ptch2 is expressed at high levels in a variety of tissues such as the somites, neural tube and the ZPA (Pearse et al., 2001). Moreover, *ptch2* expression is upregulated by Shh recombinant protein and downregulated in the presence of 5E1 Shh blocking antibody. Thus, in the chick and opposing to the mouse, Ptch2 is a transcriptional target of the

pathway and is activated even in the presence of low Shh levels, constituting a good readout of the pathway (Pearse et al., 2001).

In the absence of Hh signaling, Ptch1 catalytically inhibits the activity of Smo, that shares some structural homology with the G protein coupled receptor family (Alcedo et al., 1996; Taipale et al., 2002). Binding of Hh to Ptch1 reduces its inhibitory activity leading to the activation of Smo, which will then transduce the Hh signal to the cytoplasm (Marigo et al., 1996a; Taipale et al., 2002). Upon Hh binding, Ptch1 mediates its internalization by endocytosis and targets it to lysosomes for degradation (Incardona et al., 2000) (Fig. 2.1B). Thus, transduction of the graded Hh signaling in the receiving cell is achieved by two membrane-bound receptor proteins, Ptch1 and Smo. Hh does not directly bind Smo but regulates its activity indirectly through Ptch1. Thus, the amount of ligand-free Ptch1 dictates the activity of the Hh pathway in a cell. It is not clear how the inhibition of Smo by Ptch occurs but it seems to involve an indirect mechanism, probably through endogenous inhibitory small molecules. This is supported by the fact that several exogenous synthetic small molecules are able to bind Smo either as agonists or antagonists. Small molecule screens allowed the identification of several synthetic Smo modulators including the steroidal alkaloid inhibitor cyclopamine and the agonist SAG (Incardona et al., 1998; Chen et al., 2002a; Chen et al., 2002b). It has been suggested that Smo activation in *Drosophila* involves a conformational switch (Zhao et al., 2007). These authors identified an autoinhibitory domain in the Smo cytoplasmic tail that in the absence of Hh keeps the protein in an inactive conformation. Hh-induced phosphorylation triggers a conformational switch such that the cytoplasmic tail becomes available for further interactions. This domain is also responsible for the protein's endocytosis and degradation (Zhao et al., 2007).

In the absence of Hh ligand Smo is kept in an unphosphorylated state by Ptch, cleared from the cell surface by endocytosis and degraded in lysosomes (Zhao et al., 2007; reviewed in Huangfu and Anderson, 2006; Varjosalo and Taipale, 2008) (Fig. 2.1C). After Hh binding, *Drosophila* Smo is phosphorylated in its C-terminal tail by the protein kinase A (PKA) and casein kinase 1 (CK1). *Drosophila* Smo C-terminal domain binds directly to the kinesin-like protein Costal 2 (Cos2), which forms a complex with several kinases such as Fused (Fu), suppressor of Fused (Sufu), PKA, glycogen synthase kinase 3 (GSK3β) and CK1 and the transcription factor Cubitus interruptus (Ci), which is in its activator full length form (CiA) (reviewed in Huangfu and Anderson, 2006; Varjosalo and Taipale, 2008). In the absence of Hh, CiA is phosphorylated first by the PKA, that functions as the priming kinase and subsequently by the remaining kinases and is then degraded by the ubiquitin E3 ligase Slimb, generating a repressor truncated form of Ci (CiR), which has an

inhibitory effect on the pathway. When Hh is present, Smo phosphorylation leads to Cos2 phosphorylation and to the release of the transcriptional activator CiA, which can enter the nucleus. In vertebrates, two Cos2 orthologs, Kif27 and Kif7, have been identified but their roles in the pathway have not yet been tested. Moreover, vertebrate Hh pathway seems to depend on Sufu, which has a minor role in *Drosophila,* whereas Fu null mice are viable and present normal Hh signaling, indicating that Fu does not play a key role in vertebrates (reviewed in Huangfu and Anderson, 2006; Varjosalo and Taipale, 2008). PKA is a conserved modulator of the pathway in vertebrates and β-TrCP is the Slimb homolog. The downstream effectors of the signaling pathway in vertebrates are the Glioma (Gli) family of zinc-finger transcription factors that are regulated similarly to Ci. Three Gli proteins with distinct activator (GliA) and repressor (GliR) activities have been described in the vertebrates. Gli1 cannot be proteolytically processed and functions only as a transcriptional activator; Gli2 is bifunctional, in its full length form is a transcriptional activator and can be processed into a repressor form; Gli3 mostly acts as a repressor, although it can also have activating effects (Ruiz i Altaba, 1999; reviewed in Ruiz i Altaba et al., 2007). Thus, in the absence of Hh signaling, *gli1* is transcriptionally silent while *gli2* and *gli3* can be expressed as cleaved repressors, silencing Hh targets; after Hh binding, *gli1* and *gli2* act as activators and *gli3* is no longer cleaved. The Gli proteins are able to form Gli-Gli complexes and act cooperatively to regulate their targets.

One particular characteristic of the vertebrate Hh pathway is that contrary to *Drosophila*, primary cilia seems to play a key role in the intracellular transduction of Hh signal. Primary cilia are generally non-motile and are present on most vertebrate cells. They posses important sensorial functionalities and in the last years have been implicated in different signaling pathways such as Hh and Wnt (reviewed in Seeley and Nachury, 2010). Mutation of several proteins required for cilia formation resulted in loss of Hh signaling, suggesting that cilia are required for the Hh pathway (Kim et al., 2009; reviewed in Goetz and Anderson, 2010). In fact, all the key components of the pathway are enriched at the cilium (see Fig. 2.1). Ptch1 and Smo show Hh-dependent trafficking in cilia: in the absence of Hh, Ptch1 localizes to the base of the cilia and Smo is not detected in this structure; upon binding of either Hh or a Smo agonist, Ptch1 is no longer present (probably because it is internalized) and Smo accumulates in the cilia (Rohatgi et al., 2007). Moreover, all Gli proteins are detected both at the tip and in the nucleus of the limb bud cilia cells while Sufu, regulator of the Gli activity, is also concentrated at the ciliary tip (Haycraft et al., 2005). In this analysis, full-length Gli forms (GliA) were always detected at the tip of the cilia and never at its base, as colocalization with the basal body marker γ-tubulin was never

observed. However, the GFP tag used is fused to the protein C-terminus and Gli processing to generate the GliR form would exclude this tag and prevent its visualization. Nevertheless, Gli3R-GFP was detected mostly in the nucleus and never in the tip (Haycraft et al., 2005). Furthermore, Gli-induced transcriptional activation in the nucleus seems to require that Gli proteins first translocate to the cilia tip and only then to the nucleus: after inhibition of the dynein motor protein, Gli2 was trapped in the cilia and Hh activation decreased (Kim et al., 2009). Kif7, the vertebrate homolog of Cos2 is also detected in the cilia and changes its subcellular localization in response to pathway activation: in the absence of the ligand, the fusion protein Kif7-eGFP is detected at the base of the cilia and moves to the tip after pathway activation (Endoh-Yamagami et al., 2009; Liem et al., 2009). Taken together, these results suggest that in order to activate the Smo-downstream signaling pathway and induce the formation of GliA proteins, the signaling components need to be translocated to the cilia tip. Kif7, a kinesin motor protein that carries cargoes towards the plus end of microtubules may be responsible for this movement. In the cilia, the minus ends of microtubules are located at the base while the plus ends localize at the tip, suggesting that Kif7 may transport itself and other cargo to the tip of the cilia (Liem et al., 2009; reviewed in Goetz and Anderson, 2010). Even though several studies seem to correlate the Hh pathway components with cilia, it remains unclear what is the precise role of this structure in Hh and whether the localization of the pathway components to cilia is strictly necessary for signaling.

A recent work has shown that long-range Shh signaling is established by the reiterated secretion of Shh from the source cells by Disp and uptake by the receiving cells through Ptch1 (Etheridge et al., 2010). Cultures of *disp1*$^{+/+}$ or *disp1*$^{-/-}$ cells expressing Shh surrounded by either wild-type, *disp1*$^{-/-}$ or *ptch1*$^{-/-}$ cells indicates that in the absence of Disp1 Shh is retained in the producing cells and that Ptch1 in the receiving cells is required to remove Shh from the synthesis sites, suggesting that both Disp1 and Ptch1 play a role in the trafficking of Shh. To test if Disp is also required in the receiving cells for the long-range signaling, *disp1*$^{+/+}$ or *disp1*$^{-/-}$ embryoid bodies (EBs) were cultured in the presence of either EBs expressing Shh or chick notochords. Even though *disp*$^{-/-}$ cells are responsive to Shh, as judged by their ability to inhibit *pax7*, the expression range of this gene is shorter than in wild-type cells and *disp*$^{-/-}$ cells are unable to induce motorneuron markers, indicating that Disp1 is also required in the receiving cells to distribute Shh (Etheridge et al., 2010). The authors further show that Disp1 is localized at the basolateral side of polarized cells. However, it has been reported that Shh is located only on the apical cell side (Chamberlain et al., 2008). This suggests that the cell-to-cell Shh transport occurs in the basolateral side of the cells and after Ptch1-mediated internalization, Shh can either be loaded for

re-secretion or it can move to the apical surface, where it initiates its response at the primary cilium (Etheridge et al., 2010) (see Fig. 2.1).

## 2.4 SONIC HEDGEHOG IN DEVELOPMENT

Shh is the most broadly expressed Hh member and has been implicated in several key events during embryonic development. It is expressed in three main embryo signaling centres: the notochord, floor plate of the neural tube and in the limb ZPA. Besides the well characterized Shh functions in patterning the vertebrate limb, mature somites and the neural tube, this signalling molecule is also involved in the patterning of other structures such as the foregut. Furthermore, Shh has been implicated in organ differentiation, for example of the lung and salivary gland, where it works mainly to regulate branching (reviewed in Ingham and McMahon, 2001).

The developing vertebrate limb is specified by signalling molecules that pattern the limb along three axes (A-P, D-V and proximodistal (P-D)), emanating from key signaling centres: the ZPA, where Shh is produced; the apical ectodermal ridge (AER), an ectodermal thickening at the limb distal tip which constitutes the source of Fgfs and Wnt signaling from the dorsal ectoderm (Fig. 2.2A). A positive feedback loop operates between ZPA and AER signaling (reviewed in Benazet and Zeller, 2009). Along the P-D axis three main skeletal elements exist: the proximal stylopod, the zeugopod and the distal autopod. The A-P axis is better distinguished at the level of the zeugopod, with the radius/ulna or tibia/fibula elements and in the autopod, where the little finger is the most posterior digit and the thumb the most anterior one (digit one). The ZPA is located in the posterior distal region of the limb bud and specifies the A-P axis through graded Shh signalling. Studies in chick and mouse limb show that Shh is sufficient to pattern the limb distal elements in a dose-dependent manner, with the most posterior digit requiring the highest concentration while digit one induction does not depend of Shh signaling (Fig. 2.2A). Implantation of either cells overexpressing Shh or beads soaked in different Shh concentrations in the anterior portion of the limb induced digit mirror-image duplications in which digit identity was specified by Shh concentration: higher doses lead to the formation of more posterior digits while lower ones induced formation of anterior digits, namely digit two (Riddle et al., 1993; Yang et al., 1997). Moreover, mutant mice for Shh present a truncated limb with only one zeugopod element and one digit, the anterior-most digit one, indicating that its formation is independent of Shh signaling (Chiang et al., 2001). It has also been shown that *gli3* inactivation leads to the formation of several additional digits, with duplications of digit five, revealing its important role in specification of

digit number and identity (Ahn and Joyner, 2004). This suggests that in the limb Shh works to counteract Gli3R activities. Indeed, the posterior-to-anterior *shh* induced activity of GliA is counteracted by an opposing Gli3R gradient, suggesting that the different digits are specified by distinct levels of Gli A and R forms (Marigo et al., 1996b; Ahn and Joyner, 2004). Thus, the most posterior digit forms in the presence of GliA and absence of GliR while anterior digit formation requires solely GliR activity. This implies that the polydactylous autopod formed in the *gli3* null mutant consist of only posterior digits, which has been shown to be the case (Ahn and Joyner, 2004). In fact, in these mutants, GliA is not counteracted by GliR and expands over most of the *gli3*$^{-/-}$ embryo autopod. Surprisingly, *gli2*$^{-/-}$ mouse embryos form five well patterned digits and this can be explained by the normal anterior-to-posterior levels of Gli3R, further supporting the importance of this gradient in digit specification.

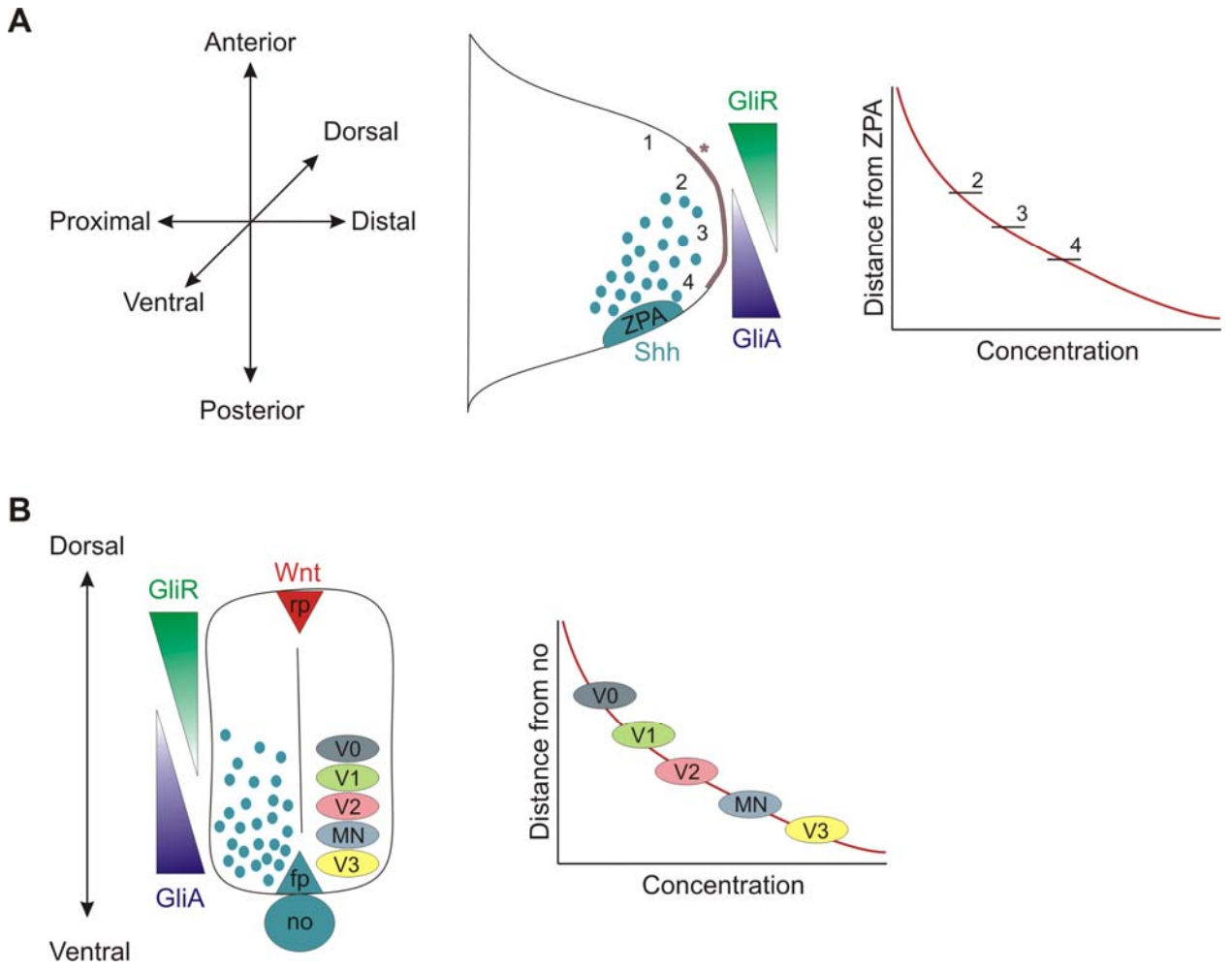


**Figure 2.2. Sonic Hedgehog patterning. (A)** Schematic representation of the outgrowing chick limb, which is patterned along its three axes, anterior-posterior, dorsal-ventral and proximal-distal. Two important signaling centers

in limb patterning are the apical ectodermal ridge (AER, darker tickening at the distal limb tip marked with *) that expresses Fgf and the zone of polarizing activity (ZPA), which is the source of Sonic hedgehog (Shh). Shh induces digit specification in a dose and time dependent manner: cells exposed to high Shh concentrations for longer periods become more posterior digits while more anterior digit creation requires progressively less Shh and digit 1 formation does not require Shh (Riddle et al., 1993; Yang et al., 1997). The gradient of activity of Shh is further shaped by the opposition of Gli transcription factors in their activator and repressor forms (GliA and GliR) because of the antagonistic effect of Shh signalling on GliR activity. Distinct digits are specified according with their progenitors' exposition to specific GliA and GliR levels (Marigo et al., 1996b; Ahn and Joyner, 2004). **(B)** Representation of ventral neural tube patterning. Shh (in blue) emanating from the notochord and the neural tube's floor plate (fp) establishes a ventral-to-dorsal protein gradient and a parallel gradient of Gli activity (GliA) (Briscoe et al., 2001). An opposing gradient of Gli proteins in their repressor forms (Gli3R) is induced by lack of Shh and Wnt signaling from the dorsal neural tube's roof plate (rp). Graded GliA and GliR transcriptional activities specify distinct cellular identities along the ventral neural tube, which will latter give rise to distinct neuronal subtypes: ventral interneuron subtypes (V0 to V3) and motorneurons (MN). The D-V position of these neurons corresponds to their requirement for increasing concentrations of Shh: more ventral progenitors emerge later and require higher Shh concentrations while progressively more anterior cells need less Shh (Ericson et al., 1996; Ericson et al., 1997).

Shh role in the midline structures, the notochord and the floor plate of the neural tube, has been well described. Shh is a key patterning signalling molecule for somite mature differentiation and formation of the distinct somitic compartments that will latter form the muscle and skeletal elements of the vertebrate body, as previously discussed in section1.5. During chick embryo somitogenesis, the Gli transcription factors are activated concomitant with somite formation (Borycki et al., 1998; Borycki et al., 2000). *Gli1* is expressed in the ventral and medial somite, preceding *pax1* and *myoD* expression; *gli2* and *gli3* are firstly activated throughout the entire somite and then become restricted to the dermamyotome. Implantation of beads soaked in ShhNp showed that *gli1* expression is induced by Shh while *gli2* and *gli3* activation does not require that signaling molecule (Borycki et al., 2000). Expression of *gli2* and *gli3* is in fact regulated by Wnt signaling from the surface ectoderm: analysis of quail PSM explants cultured either in the presence of a fibroblast line expressing Wnt or with LiCl (which mimics Wnt signaling by inhibiting GSK3β and thus increasing β-catenin levels), shows that Wnt induces *gli2* and represses *gli3*. This indicates that during somite formation, derepression of *gli3* by the Wnt pathway must occur.

The effect of Shh dosage in patterning target tissues is best characterized in cell identity specification in the ventral neural tube. The notochord and the floor plate of the neural tube derive from the HN (Catala et al., 1996) and both express Shh, creating a protein concentration gradient

across the ventral neural tube that specifies different neuronal subtypes at defined positions (reviewed in Jacob and Briscoe, 2003; Dessaud et al., 2008) (Fig. 2.2B). The confirmation that Shh acts directly on the neural tube to pattern it came from the observation that expression of a deleted form of Ptch1 that does not bind Shh but is able to bind and inhibit Smo ($Ptch1^{\Delta loop2}$), results in a ventral-to-dorsal switch in progenitor identity (Briscoe et al., 2001). The generation of mice producing Shh fused to GFP (Shh-GFP) allowed the direct analysis of Shh protein distribution along the mouse neural tube, showing a clear ventral-to-dorsal protein gradient and revealing its long range spread (Chamberlain et al., 2008). In the responding cells, Shh regulates the Gli transcription factors increasing their activator function, which induce the expression of several transcription factors that subdivide the neural tube into five molecularly distinct subpopulations of neuron progenitors. Thus, each progenitor's domain is characterized by the expression of a specific combination of transcription factors, which determines a distinct neuronal subtype progeny forming different ventral interneuron subtypes (V0 to V3) or motorneurons (MN) (reviewed in Jacob and Briscoe, 2003; Dessaud et al., 2008). The order of appearance and the final position of these progenitor domains corresponds to their requirement for increasing concentrations of Shh: more ventral progenitors emerge later and require higher Shh concentrations (Ericson et al., 1996; Ericson et al., 1997). Chick neural plate explants cultured in the presence of ShhNp formed all neuronal subtypes in a dose-dependent manner: V1 required the lowest concentration, V2 a two-fold higher dose and MN another two-fold Shh amount (Ericson et al., 1997). Thus, along the D-V neural tube axis, the floor plate is found in the most ventral position, followed by V3, MN, V2, V1 and V0 interneurons in the most dorsal location (Fig. 2.2B). As in the limb, the balance between Gli activator and repressor forms is responsible for the specification of different neuronal types. In fact, both in mouse and chick embryos, *gli1* is expressed in the more ventral portion of the neural tube, *gli2* is present in the medial region and *gli3* is detected in the dorsal-most neural tube domain (Lee et al., 1997; Borycki et al., 2000). Injection of *gli1* in zebrafish forebrain induces the expression of *nkx2.2*, a transcription factor normally expressed in the ventral neural tube in response to Shh signaling (Lee et al., 1997). *Gli3*, however, is unable to induce ventral neural tube development, suggesting that it acts negatively on Shh signaling. Accordingly, mice lacking *gli3* do not form the dorsal progenitors and present a dorsal expansion of the intermediate transcription factors *nkx6.2*, *dbx2* and *dbx1* (Persson et al., 2002). Furthermore, these authors show that mice encoding a truncated form of Gli3, similar to the processed Gli3R form, present a normal patterning of the neural tube highlighting the importance of Gli3R for correct neural tube development. On the other hand, *gli2* null mice present reduced

*gli1* and *ptch1* expression and fail to develop a floor plate but still present MN, which are located ventrally in the neural tube (Ding et al., 1998; Matise et al., 1998) while *gli1* mutant mice do not present any obvious phenotype (Matise et al., 1998). Taken together, *gli2* seems to be required for the transduction of high Shh levels in the most ventral neural tube while Gli3R is responsible for inducing more dorsal fates (Wijgerde et al., 2002). In fact, the loss of ventral neurons in *shh* and *smo* mutant mice is rescued in the double *shh*/*gli3* and *smo*/*gli3* mutants (Wijgerde et al., 2002 and see Persson et al., 2002 and references therein), supporting an antagonistic relation between Shh and Gli3 to pattern the neural tube. However, data from the double mutants *gli2*/*gli3* seem to indicate that these two transcription factors cooperate to induce ventral differentiation (Lei et al., 2004). These authors generated Gli2 and Gli3 activator constructs by deleting the N-terminal repressor domain region. Electroporation of these constructs in chick embryos induced the expression of *ptch1* and ventral markers, indicating that those genes present redundant functions in inducing V3 interneurons (Lei et al., 2004). Moreover, it has been shown that a gradient of Gli transcriptional activity is sufficient to regulate neural tube patterning, even in the absence of endogenous Shh signaling (Stamataki et al., 2005). By deleting part or the entire N-terminal repressor domain, these authors generated three Gli3A constructs with distinct efficiency in Shh pathway activation – low, medium and high, which are responsible for inducing specific transcription factor activation in the dorsal, intermediate and ventral neural tube, respectively. Co-expression of $Gli3^{high}$ or $Gli3^{med}$ with $Ptch1^{\Delta loop2}$, a cell-autonomous Shh inhibitor, induced the same patterning as the Gli3A constructs. This indicates that the specification induced by these constructs is cell autonomous and does not depend on an ectopic activation of the pathway.

## 2.5 MODULATION OF THE HEDGEHOG GRADIENT

The Hh pathway is complex, with many proteins and specific components and thus presents numerous putative mechanisms of modulation that control the size and shape of the Hh gradient (reviewed in Dessaud et al., 2008; Varjosalo and Taipale, 2008). These mechanisms are also important for the generation of multiple responses to a single morphogen (see Fig. 2.3). The spread of Shh ligand is primarily regulated by the proteins involved in its processing and secretion, as discussed in section 2.2. For example, *skn* mutants present a dramatic reduction in Shh protein levels, reflecting a failure of cell-surface proteins to bind or traffic the non-palmitoylated ligand (Chamberlain et al., 2008). The components of its signaling cascade can also influence Shh diffusion. Smo, for instance, can induce distinct cell responses according to its degree of activation

(Hooper, 2003). In the absence of Hh (the off state), Smo is not activated and repressor forms of the downstream effectors are generated. When Hh is available in low concentrations (low state), Cos2 is able to bind the cytoplasmic tail of Smo while Sufu and Fu still bind Ci, leading to the production of low levels of CiA and CiR. Finally, when Hh levels are high (high state), the Cos2/Fu/Sufu complex bind Smo and high doses of CiA are produced. This model was further supported by the observation that increased Hh levels induce increasingly higher Smo phosphorylation, which leads to a progressive increase of Smo expression and activity in the cell surface (Zhao et al., 2007). Thus, Smo activity can be translated into graded Hh signal and distinct cell responses. In fact, in Smo null mutants, which lack a Shh response, Shh-GFP gradient is less restricted and spans over longer distances in the neural tube (Chamberlain et al., 2008).

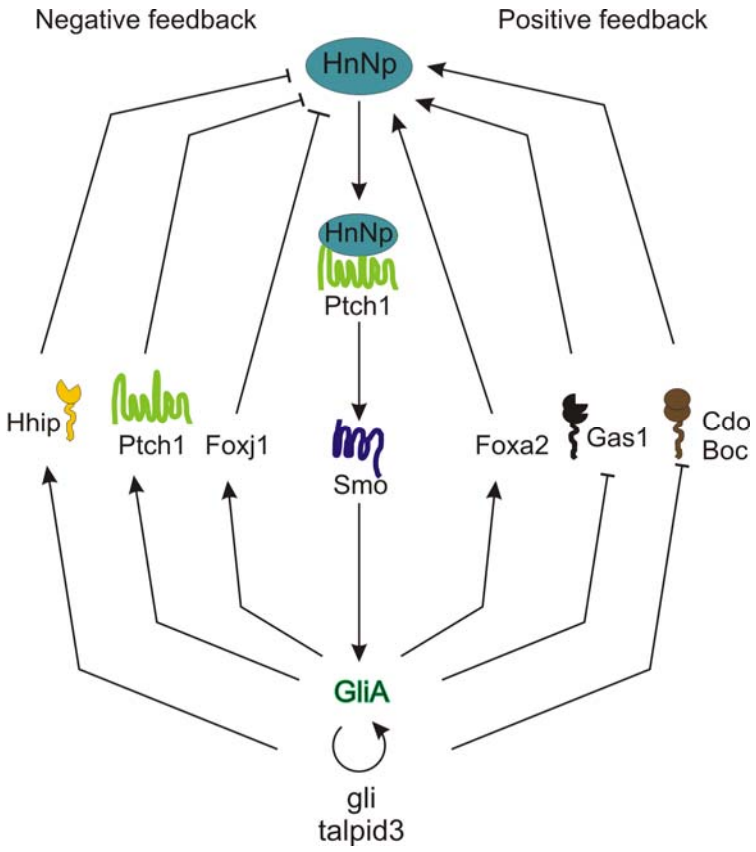


**Figure 2.3. Feedback loops modulating the Hedgehog pathway.** The Hedgehog (HhNp) gradient of activity is dynamically regulated by several proteins through positive and negative feedback loops. Examples of some of these mechanisms are depicted. Activated Smoothened (Smo) triggers the formation of Gli proteins in their active form (GliA), which regulate the expression of several Hh targets. Increasing phosphorylation of Smo further activates this protein and the downstream signaling pathway (Hooper, 2003; Zhao et al., 2007). Gli protein levels are controlled by other Gli proteins and by Talpid3 (Davey et al., 2006). Gas1, Cdo and Boc are transmembranar proteins repressed by the Hh pathway but that act to induce the pathway probably by binding directly to Shh and facilitating its presentation

to Patched1 (Ptch1) (Tenzen et al., 2006; Allen et al., 2007). On the other hand, Ptch1 and hedgehog interacting protein (Hhip) are negative regulators of the Hh pathway. They are upregulated by GliA and bind and sequester Hh ligand, decreasing its availability and reducing its spread range (Chuang and McMahon, 1999; Jeong and McMahon, 2005).

Another level of Hh regulation is provided by the genes induced in target tissues, which can be involved in positive and negative feedbacks to the pathway itself and regulate the activity and spread of the ligand. Genes that are transcriptionally upregulated by Hh and induce a cell-autonomous inhibition of the pathway include *ptch1* and the *hedgehog-interacting protein* (*Hhip*), which encodes a membrane-linked protein. Both genes bind Hh reducing its movement range and *ptch1* further induces endocytosis and degradation of the ligand (Chen and Struhl, 1996; Chuang and McMahon, 1999; Incardona et al., 2000; Jeong and McMahon, 2005). This kind of regulation is designated ligand-dependent antagonism (LDA) and diminishes the availability of the ligand, which attenuates Hh signal range and pathway activity thus sharpening the gradient. LDA induces a cell-autonomous decreased sensitivity for the ligand and therefore leads to non cell-autonomous consequences in the target field (Jeong and McMahon, 2005). This regulation is required for the correct formation of all types of ventral neurons in the neural tube: removing the negative feedback mediated by these genes in double null mouse mutants for *ptch1* and *Hhip*, caused an expansion of ventral neural progenitors, indicating that the Hh pathway range was abnormally increased (Jeong and McMahon, 2005). Another group of genes that are able to bind Hh and enhance the pathway through positive feedback loops include the transmembranar proteins *cdo* (or *cdon*), *boc* (Tenzen et al., 2006) and *growth arrest specific gene 1* (*gas1*) (Allen et al., 2007). Shh signaling negatively regulates the expression of *gas1*, *cdo* and *boc* and ectopic expression of these genes in the chick neural tube induced ventral neuronal identities (Tenzen et al., 2006; Allen et al., 2007). Coelectroporation of these genes with the Ptch1$^{\Delta loop2}$ construct (Briscoe et al., 2001) prevented the induction of ventral neurons, indicating that *gas1*, *cdo* and *boc* proteins are able to directly bind Shh ligand, probably to facilitate the presentation of Shh to Ptch1 (Tenzen et al., 2006).

Analysis of the transcriptional regulators acting upstream of *shh* have allowed the identification of enhancers directing the expression of *shh* in specific regions of the mouse body (Epstein et al., 1999). However, the specific transcription factors that control the enhancer's activity have not been completely identified. Among them, is the *forkhead box a2* gene (*foxa2*), also known as *hepatocyte nuclear factor 3β* (*hnf3β*), which is a downstream target of the Hh

pathway and can induce *shh* expression both in zebrafish, mouse and chicken (Chang et al., 1997; Epstein et al., 1999; Ribes et al., 2010). This gene is necessary for neural tube floor plate induction and maintenance: first, it is induced by high Shh levels coming from the notochord and later *foxa2* specifies the floor plate identity independently of Shh signaling (Ribes et al., 2010). Moreover, a retinoic acid response element (RARE) has been identified in the *shh* promoter and HeLa cells tranfected with RARβ and treated with all-*trans* RA presented increased Hh activity, indicating that *shh* is transcriptionally regulated by RARs (Chang et al., 1997). In fact, *raldh2* null embryos present a decreased Shh signaling efficiency as evidenced by the diminished *ptch1* and *gli1* expression, both direct Shh target genes (Ribes et al., 2006; Ribes et al., 2009). Additionally, a recent screen in neural cells in which Shh signaling had been either constitutively inhibited or activated allowed the identification of several genes regulated by Shh (Cruz et al., 2010). In the cluster of genes upregulated by Shh is the forkhead transcription factor *foxj1* that is associated with the production of motile cilia and expressed in the floor plate of mouse and chick embryos. Ectopic expression of *foxj1* in the neural tube attenuates Shh signaling, as evidenced by a decrease in *ptch1* and *gli1* expression and this is probably mediated by a defective localization of Gli2 in the cilia: even though Smo traffic in the cilia appears normal, there is an accumulation of Gli2 at the cilia tip (Cruz et al., 2010). This data evidences a new level of Shh signaling modulation and interpretation, the cilia, thus bringing even more complexity to the regulation of this pathway.

The existence of three Gli proteins with distinct activities provides an additional mechanism of regulation and complexity of the pathway. In fact, these proteins can regulate each other and have been shown to be regulated by different genes (reviewed in Ruiz i Altaba et al., 2002; Ruiz i Altaba et al., 2007). For example, *gli1* can inhibit *gli3* whereas *gli2* is able to induce *gli1*. Moreover, Wnt signaling has been shown to induce *gli2* and repress *gli3* during somite formation (Borycki et al., 2000). In the developing neural tube Wnt signaling induces Gli3R, helping to restrict the graded Gli activity in the ventral neural tube (Alvarez-Medina et al., 2008). The gene *talpip3* was shown to be necessary for proper Gli processing and thus to generate the correct GliA/GliR ratio in the chick (Davey et al., 2006). Interestingly, recently this gene has been associated with cilia formation and chick embryos lacking the *talpid3* gene loose cilia in the neural tube lumen, notochord, somites and limb bud mesenchymal cells (Yin et al., 2009). The fact that other signaling pathways can regulate Gli activity suggests that either there is an Hh-independent Gli activity or that Shh acts in coordination with other signaling pathways to regulate *gli* expression in specific tissues. This is further complicated by the suggestion of the existence of

non-canonical Hh signaling pathways, which can culminate in Gli-independent Shh-mediated transcriptional regulation (reviewed in Jenkins, 2009).

All the data presented here points to a complex regulation of the Hh signaling gradient, which is modified and sharpened by feedback regulations along the target cell field. A model for this regulation can therefore be proposed: molecules capable of promoting Shh signaling (e.g. Gas1, Cdo, Boc) are initially expressed in responsive cells to sensitize them to low ligand levels; consequently, activation of the pathway leads to their transcriptional downregulation and an upregulation of the negative feedback components (*ptch1* and *Hhip*) (Allen et al., 2007). Therefore, over time cells become progressively desensitized to the avaiable Shh protein. This dynamic regulation of the pathway provides not only positional information to the cells but also a notion of duration of exposure and a given Shh concentration can be translated into a proportional period of Gli activity.

## 2.6 DOSE- AND TIME- DEPENDENT HEDGEHOG RESPONSE

During embryogenesis, the Hh pathway operates in the patterning of several tissues and inducing distinct developmental processes. The ability of a single morphogen to affect such a variety of tissues during vertebrate development relies on a well regulated signaling cascade with different feedback control mechanisms, which together create a robust and sharp morphogenetic gradient. As previously discussed, it is widely accepted that the cellular response to Hh depends not only on the ligand but also on the characteristics of the receiving cell and on the dose to which this cell is exposed. A good example of this is the ventral neural tube, where different Shh ligand concentrations induce distinct neural identities. However, the Hh-dependent patterning does not emerge at a single time point but occurs over time. Therefore, the time of exposure to the Hh signal also seems influence the cell's response. This has been clearly shown to occur during both digit and neuronal specification.

In the limb, a temporal gradient model states that the time spent sensing Shh provides cells with a kinetic memory relevant to the specification of their A-P identities: more anterior digits are formed first by cells that were under Shh influence for a short time period whereas the progenitors of digits 4 and 5 contain cells that were exposed to high Shh concentration for progressively longer periods of time. This was shown by implanting Shh-soaked beads in the anterior margin of the chick limb bud and removing it after different time periods (Yang et al., 1997). When beads were removed after 16h of incubation, an additional digit 2 was formed and progressively longer

incubation periods induced the formation of more posterior digits so that after 24h of treatment, an additional digit 4 was formed. Moreover, a higher Shh concentration applied for a shorter time can produce the same results as incubation with a lower concentration for a longer period (Yang et al., 1997). In fact, in the neural tube it has been shown that changing the concentration or the duration of Shh exposure has an equivalent effect on the intracellular signaling (Stamataki et al., 2005). This happens because different ligand concentrations are converted into time periods of signaling transduction, a mechanism designated as temporal adaptation (Dessaud et al., 2007; Dessaud et al., 2010). Through Gli activity measurements using a luciferase reporter plasmid, the authors show that induction of a specific D-V identity requires the maintenance of Gli activity for a determined period of time. Exposure of neural tube explants to different doses of ShhNp, induces steady Gli levels for 12h after which they decrease over time with a rate inversely proportional to Shh concentration. Addition of cyclopamine, an antagonist of the Hh pathway, after 12h, leads to a failure in inducing the subsequent transcription factors, demonstrating that the duration of the Gli activity is crucial for the correct induction of transcription factors along the neural tube (Dessaud et al., 2007). In fact, a basal Gli activity is necessary for ventral cells to maintain their identity (Dessaud et al., 2010). Blockage of Shh signaling at a time point when progenitor domains are already established using either cyclopamine, Ptch1$^{\Delta loop2}$ or mutant mice results in acquisition of a more dorsal fate (Dessaud et al., 2010). The decrease in Gli activity over time even in the presence of high Shh levels seems to indicate the existence of an adaptation mechanism of the neural tube cells, which become desensitized to Shh signaling. In fact, small interfering RNAs (siRNAs) to block the Shh-induced *ptch1* upregulation, disrupted the cells ability to interpret Shh concentrations and ventral neural tube specification, indicating that Ptch1 is required for this regulation (Dessaud et al., 2007). These results indicate that cells exposed to a Shh gradient acquire their positional information based on both the morphogen concentration to which they are exposed and the duration of the exposure. Ptch1 plays a key role in the morphogen interpretation by the morphogenetic field cells: its feedback action influences both the spread and duration of the signal allowing the integration of concentration and timing information received by each cell. The transduction of a morphogen concentration into transcriptional duration is an additional mechanism to establish a robust graded signaling.

## 2.7 SONIC HEDGEHOG AND DISEASE

Embryo development is regulated by several signaling pathways, being one of them the Hh pathway. It has been shown that disruption of Hh components during human embryo development leads to several developmental defects while aberrant signaling in postnatal life induces uncontrolled cell growth and cancers (reviewed in Villavicencio et al., 2000; Varjosalo and Taipale, 2008). Mutations in the human *shh* or its downstream effectors *gli2* and *gli3* causes disorders such as holoprosencephaly, severe midline defects that includes cleft lip and palate, cyclopia, craniofacial defects, skeletal malformations and polydactyly reviewed in Kim et al., 2001; Celli et al., 2003). Mutations on *ptch1* or *smo* have been associated with basal cell carcinomas, the most common type of skin cancer and medulloblastomas. *Gli1* overexpression is found in glioblastomas and B cell lymphomas and the degree of overexpression has been related with tumor grade. Moreover, mouse models for ciliopathies, syndromes associated with the disruption of primary cilia, are now starting to bring further insights on cilia and Hh signaling (reviewed in Simpson et al., 2009). The Hh pathway has been shown to control proliferation and growth in a variety of cell and tissue types and the molecular mechanisms by which this regulation occurs are now the focus of many studies. A better comprehension of Hh pathway role and regulation in embryonic processes such as cell proliferation and survival, tissue patterning and identity specification is crucial for the understanding of the Hh-related diseases and determination of potential therapeutic approaches.

# 3. AIMS AND THESIS LAYOUT

Somitogenesis is a periodic highly controlled mechanism. Underlying this periodicity is an intrinsic molecular oscillator designated segmentation molecular clock that includes members of the Fgf, Wnt and Notch signaling pathways. Morphological somite formation has been described to depend on fibronectin produced by the overlying ectoderm (Rifes et al., 2007). An intriguing report, however, showed that grafting of a quail PSM into a chick embryo induces the progressive adjustment of somite formation to the host PSM, strongly suggesting that a signal from the embryo midline structures is required to bilaterally adjust somite formation (Packard et al., 1993). Molecular clock oscillations have been considered to be an intrinsic property of the PSM, occurring independently of the surrounding tissues (Palmeirim et al., 1997). However, isolated lateral PSM loses not only the ability to segment but also the expression of molecular clock

components (Freitas et al., 2001), suggesting that medial and lateral PSM domains present distinct intrinsic properties. Sonic hedgehog (Shh) is a morphogen produced by the embryo axial structures, which has been shown to regulate multiple developmental processes (reviewed in Varjosalo and Taipale, 2008). In fact, Shh knock-out mice lack the entire vertebrate column except for five to six ribs (Chiang et al., 1996).

Therefore, the aim of this thesis was to evaluate the contribution of the notochord and Shh signaling in somite formation and molecular clock oscillations. We have also analyzed the role of Shh in the differential specification of medial and lateral PSM tissue. The understanding of the molecular mechanisms underlying vertebrate embryo segmentation is still limited and with this work we implicate Shh as a new molecular player in this regulatory network, bringing forward new insight into this subject.

**Chapter 2** contains results already published in *Proc Natl Acad Sci U S A* (2010) 107(29):12907-12, implicating notochord-derived Shh signaling in somite formation and molecular clock oscillations regulation. We propose a model where Shh acts together with RA to ensure timely somite formation. **Chapter 3** presents a manuscript in preparation for publication evaluating Shh role in medial and lateral PSM specification. The results here shown indicate that Shh is necessary for medial and lateral PSM cell fate determination, further implicating Shh in somitogenesis regulation. Finally, in **Chapter 4** the main results obtained in previous chapters are integrated and the significance of the results presented in this thesis is subject to overall discussion. A model for the Shh signaling activity in the PSM is presented.

## 4. REFERENCES

Ahn, S. and Joyner, A. L. (2004) 'Dynamic changes in the response of cells to positive hedgehog signaling during mouse limb patterning', *Cell* 118(4): 505-16.

Alcedo, J., Ayzenzon, M., Von Ohlen, T., Noll, M. and Hooper, J. E. (1996) 'The Drosophila smoothened gene encodes a seven-pass membrane protein, a putative receptor for the hedgehog signal', *Cell* 86(2): 221-32.

Allen, B. L., Tenzen, T. and McMahon, A. P. (2007) 'The Hedgehog-binding proteins Gas1 and Cdo cooperate to positively regulate Shh signaling during mouse development', *Genes Dev* 21(10): 1244-57.

Alvarez-Medina, R., Cayuso, J., Okubo, T., Takada, S. and Marti, E. (2008) 'Wnt canonical pathway restricts graded Shh/Gli patterning activity through the regulation of Gli3 expression', *Development* 135(2): 237-47.

Andrade, R. P., Palmeirim, I. and Bajanca, F. (2007) 'Molecular clocks underlying vertebrate embryo segmentation: A 10-year-old hairy-go-round', *Birth Defects Res C Embryo Today* 81(2): 65-83.

Aulehla, A. and Johnson, R. L. (1999) 'Dynamic expression of lunatic fringe suggests a link between notch signaling and an autonomous cellular oscillator driving somite segmentation', *Dev.Biol.* 207(1): 49-61.

Aulehla, A. and Pourquie, O. (2010) 'Signaling gradients during paraxial mesoderm development', *Cold Spring Harb Perspect Biol* 2(2): a000869.

Aulehla, A., Wehrle, C., Brand-Saberi, B., Kemler, R., Gossler, A., Kanzler, B. and Herrmann, B. G. (2003) 'Wnt3a plays a major role in the segmentation clock controlling somitogenesis', *Dev Cell* 4(3): 395-406.

Aulehla, A., Wiegraebe, W., Baubet, V., Wahl, M. B., Deng, C., Taketo, M., Lewandoski, M. and Pourquie, O. (2008) 'A beta-catenin gradient links the clock and wavefront systems in mouse embryo segmentation', *Nat Cell Biol* 10(2): 186-93.

Barrios, A., Poole, R. J., Durbin, L., Brennan, C., Holder, N. and Wilson, S. W. (2003) 'Eph/Ephrin signaling regulates the mesenchymal-to-epithelial transition of the paraxial mesoderm during somite morphogenesis', *Curr Biol* 13(18): 1571-82.

Benazeraf, B., Francois, P., Baker, R. E., Denans, N., Little, C. D. and Pourquie, O. (2010) 'A random cell motility gradient downstream of FGF controls elongation of an amniote embryo', *Nature* 466(7303): 248-52.

Benazet, J. D. and Zeller, R. (2009) 'Vertebrate limb development: moving from classical morphogen gradients to an integrated 4-dimensional patterning system', *Cold Spring Harb Perspect Biol* 1(4): a001339.

Bessho, Y., Hirata, H., Masamizu, Y. and Kageyama, R. (2003) 'Periodic repression by the bHLH factor Hes7 is an essential mechanism for the somite segmentation clock', *Genes Dev* 17(12): 1451-6.

Bessho, Y., Miyoshi, G., Sakata, R. and Kageyama, R. (2001a) 'Hes7: a bHLH-type repressor gene regulated by Notch and expressed in the presomitic mesoderm', *Genes Cells* 6(2): 175-85.

Bessho, Y., Sakata, R., Komatsu, S., Shiota, K., Yamada, S. and Kageyama, R. (2001b) 'Dynamic expression and essential functions of Hes7 in somite segmentation', *Genes Dev* 15(20): 2642-7.

Bettenhausen, B., Hrabe de Angelis, M., Simon, D., Guenet, J. L. and Gossler, A. (1995) 'Transient and restricted expression during mouse embryogenesis of Dll1, a murine gene closely related to Drosophila Delta', *Development* 121(8): 2407-18.

Bornemann, D. J., Duncan, J. E., Staatz, W., Selleck, S. and Warrior, R. (2004) 'Abrogation of heparan sulfate synthesis in Drosophila disrupts the Wingless, Hedgehog and Decapentaplegic signaling pathways', *Development* 131(9): 1927-38.

Borycki, A.-G., Brown, A. M. C. and Emerson, J. C. P. (2000) 'Shh and Wnt signaling pathways converge to control Gli gene activation in avian somite', *Development* 127: 2075-2087.

Borycki, A. G., Mendham, L. and Emerson, C. P., Jr. (1998) 'Control of somite patterning by Sonic hedgehog and its downstream signal response genes', *Development* 125(4): 777-790.

Bottcher, R. T. and Niehrs, C. (2005) 'Fibroblast growth factor signaling during early vertebrate development', *Endocr Rev* 26(1): 63-77.

Brent, A. E., Schweitzer, R. and Tabin, C. J. (2003) 'A somitic compartment of tendon progenitors', *Cell* 113(2): 235-48.

Briscoe, J., Chen, Y., Jessell, T. M. and Struhl, G. (2001) 'A Hedgehog-Insensitive Form of Patched Provides Evidence for Direct Long-Range Morphogen Activity of Sonic Hedgehog in the Neural Tube', *Molecular Cell* 7: 1279-1291.

Buchberger, A., Seidl, K., Klein, C., Eberhardt, H. and Arnold, H. H. (1998) 'cMeso-1, a novel bHLH transcription factor, is involved in somite formation in chicken embryos', *Dev.Biol.* 199(2): 201-215.

Burgess, R., Rawls, A., Brown, D., Bradley, A. and Olson, E. N. (1996) 'Requirement of the paraxis gene for somite formation and musculoskeletal patterning', *Nature* 384(6609): 570-3.

Bussen, M., Petry, M., Schuster-Gossler, K., Leitges, M., Gossler, A. and Kispert, A. (2004) 'The T-box transcription factor Tbx18 maintains the separation of anterior and posterior somite compartments', *Genes Dev* 18(10): 1209-21.

Cambray, N. and Wilson, V. (2007) 'Two distinct sources for a population of maturing axial progenitors', *Development* 134(15): 2829-40.

Carpenter, D., Stone, D. M., Brush, J., Ryan, A., Armanini, M., Frantz, G., Rosenthal, A. and de Sauvage, F. J. (1998) 'Characterization of two patched receptors for the vertebrate hedgehog protein family', *Proc Natl Acad Sci U S A* 95(23): 13630-4.

Catala, M., Teillet, M. A., De Robertis, E. M. and Le Douarin, M. L. (1996) 'A spinal cord fate map in the avian embryo: while regressing, Hensen's node lays down the notochord and floor plate thus joining the spinal cord lateral walls', *Development* 122(9): 2599-610.

Catala, M., Teillet, M. A. and Le Douarin, N. M. (1995) 'Organization and development of the tail bud analyzed with the quail-chick chimaera system', *Mech Dev* 51(1): 51-65.

Celli, J., van Bokhoven, H. and Brunner, H. G. (2003) 'Feingold syndrome: clinical review and genetic mapping', *Am J Med Genet A* 122A(4): 294-300.

Chamberlain, C. E., Jeong, J., Guo, C., Allen, B. L. and McMahon, A. P. (2008) 'Notochord-derived Shh concentrates in close association with the apically positioned basal body in neural target cells and forms a dynamic gradient during neural patterning', *Development* 135(6): 1097-106.

Chang, B. E., Blader, P., Fischer, N., Ingham, P. W. and Strahle, U. (1997) 'Axial (HNF3beta) and retinoic acid receptors are regulators of the zebrafish sonic hedgehog promoter', *EMBO J* 16(13): 3955-64.

Chen, J. K., Taipale, J., Cooper, M. K. and Beachy, P. A. (2002a) 'Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened', *Genes Dev* 16(21): 2743-8.

Chen, J. K., Taipale, J., Young, K. E., Maiti, T. and Beachy, P. A. (2002b) 'Small molecule modulation of Smoothened activity', *Proc Natl Acad Sci U S A* 99(22): 14071-6.

Chen, L. and Deng, C. X. (2005) 'Roles of FGF signaling in skeletal development and human genetic diseases', *Front Biosci* 10: 1961-76.

Chen, M. H., Li, Y. J., Kawakami, T., Xu, S. M. and Chuang, P. T. (2004) 'Palmitoylation is required for the production of a soluble multimeric Hedgehog protein complex and long-range signaling in vertebrates', *Genes Dev* 18(6): 641-59.

Chen, Y. and Struhl, G. (1996) 'Dual Roles for Patched in Sequestering and Transducing Hedgehog', *Cell* 87: 553-563.

Chiang, C., Litingtung, Y., Harris, M. P., Simandl, B. K., Li, Y., Beachy, P. A. and Fallon, J. F. (2001) 'Manifestation of the limb prepattern: limb development in the absence of sonic hedgehog function', *Dev Biol* 236(2): 421-35.

Chiang, C., Litingtung, Y., Lee, E., Young, K. E., Corden, J. L., Westphal, H. and Beachy, P. A. (1996) 'Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function', *Nature* 383(6599): 407-13.

Christ, B., Huang, R. and Wilting, J. (2000) 'The development of the avian vertebral column', *Anat Embryol (Berl)* 202(3): 179-94.

Christ, B. and Ordahl, C. P. (1995) 'Early stages of chick somite development', *Anat.Embryol.(Berl)* 191(5): 381-396.

Christ, B., Schmidt, C., Huang, R., Wilting, J. and Brand-Saberi, B. (1998) 'Segmentation of the vertebrate body', *Anat.Embryol.(Berl)* 197(1): 1-8.

Chuang, P. T. and McMahon, A. P. (1999) 'Vertebrate Hedgehog signalling modulated by induction of a Hedgehog-binding protein', *Nature* 397(6720): 617-21.

Cole, S. E., Levorse, J. M., Tilghman, S. M. and Vogt, T. F. (2002) 'Clock regulatory elements control cyclic expression of Lunatic fringe during somitogenesis', *Dev Cell* 3(1): 75-84.

Collier, J. R., McInerney, D., Schnell, S., Maini, P. K., Gavaghan, D. J., Houston, P. and Stern, C. D. (2000) 'A cell cycle model for somitogenesis: mathematical formulation and numerical simulation', *J Theor Biol* 207(3): 305-16.

Cooke, J. and Zeeman, E. C. (1976) 'A clock and wavefront model for control of the number of repeated structures during animal morphogenesis', *J.Theor.Biol.* 58(2): 455-476.

Crossley, P. H. and Martin, G. R. (1995) 'The mouse Fgf8 gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo', *Development* 121(2): 439-51.

Cruz, C., Ribes, V., Kutejova, E., Cayuso, J., Lawson, V., Norris, D., Stevens, J., Davey, M., Blight, K., Bangs, F. et al. (2010) 'Foxj1 regulates floor plate cilia architecture and modifies the response of cells to sonic hedgehog signalling', *Development* 137(24): 4271-82.

Dale, J. K., Malapert, P., Chal, J., Vilhais-Neto, G., Maroto, M., Johnson, T., Jayasinghe, S., Trainor, P., Herrmann, B. and Pourquie, O. (2006) 'Oscillations of the snail genes in the presomitic mesoderm coordinate segmental patterning and morphogenesis in vertebrate somitogenesis', *Dev Cell* 10(3): 355-66.

Dale, J. K., Maroto, M., Dequéant, M. L., Malapert, P., McGrew, M. and Pourquie, O. (2003) 'Periodic notch inhibition by lunatic fringe underlies the chick segmentation clock', *Nature* 421(6920): 275-8.

Davey, M. G., Paton, I. R., Yin, Y., Schmidt, M., Bangs, F. K., Morrice, D. R., Smith, T. G., Buxton, P., Stamataki, D., Tanaka, M. et al. (2006) 'The chicken talpid3 gene encodes a novel protein essential for Hedgehog signaling', *Genes Dev* 20(10): 1365-77.

Delfini, M. C., Dubrulle, J., Malapert, P., Chal, J. and Pourquie, O. (2005) 'Control of the segmentation process by graded MAPK/ERK activation in the chick embryo', *Proc Natl Acad Sci U S A* 102(32): 11343-8.

Dequéant, M. L., Glynn, E., Gaudenz, K., Wahl, M., Chen, J., Mushegian, A. and Pourquie, O. (2006) 'A complex oscillating network of signaling genes underlies the mouse segmentation clock', *SCIENCE* 314(5805): 1595-8.

Dessaud, E., McMahon, A. P. and Briscoe, J. (2008) 'Pattern formation in the vertebrate neural tube: a sonic hedgehog morphogen-regulated transcriptional network', *Development* 135(15): 2489-503.

Dessaud, E., Ribes, V., Balaskas, N., Yang, L. L., Pierani, A., Kicheva, A., Novitch, B. G., Briscoe, J. and Sasai, N. (2010) 'Dynamic assignment and maintenance of positional identity in the ventral neural tube by the morphogen sonic hedgehog', *PLoS Biol* 8(6): e1000382.

Dessaud, E., Yang, L. L., Hill, K., Cox, B., Ulloa, F., Ribeiro, A., Mynett, A., Novitch, B. G. and Briscoe, J. (2007) 'Interpretation of the sonic hedgehog morphogen gradient by a temporal adaptation mechanism', *Nature* 450(7170): 717-20.

Diez del Corral, R., Olivera-Martinez, I., Goriely, A., Gale, E., Maden, M. and Storey, K. (2003) 'Opposing FGF and retinoid pathways control ventral neural pattern, neuronal differentiation, and segmentation during body axis extension', *Neuron* 40(1): 65-79.

Ding, Q., Motoyama, J., Gasca, S., Mo, R., Sasaki, H., Rossant, J. and Hui, C. C. (1998) 'Diminished Sonic hedgehog signaling and lack of floor plate differentiation in Gli2 mutant mice', *Development* 125(14): 2533-43.

Dockter, J. and Ordahl, C. P. (2000) 'Dorsoventral axis determination in the somite: a re-examination', *Development* 127(10): 2201-6.

Duband, J. L., Dufour, S., Hatta, K., Takeichi, M., Edelman, G. M. and Thiery, J. P. (1987) 'Adhesion molecules during somitogenesis in the avian embryo', *J Cell Biol* 104(5): 1361-74.

Dubrulle, J., McGrew, M. J. and Pourquie, O. (2001) 'FGF signaling controls somite boundary position and regulates segmentation clock control of spatiotemporal Hox gene activation', *Cell* 106(2): 219-232.

Dubrulle, J. and Pourquie, O. (2004) 'fgf8 mRNA decay establishes a gradient that couples axial elongation to patterning in the vertebrate embryo', *Nature* 427(6973): 419-22.

Dunty, W. C., Jr., Biris, K. K., Chalamalasetty, R. B., Taketo, M. M., Lewandoski, M. and Yamaguchi, T. P. (2008) 'Wnt3a/beta-catenin signaling controls posterior body development by coordinating mesoderm formation and segmentation', *Development* 135(1): 85-94.

Dunwoodie, S. L., Henrique, D., Harrison, S. M. and Beddington, R. S. (1997) 'Mouse Dll3: a novel divergent Delta gene which may complement the function of other Delta homologues during early pattern formation in the mouse embryo', *Development* 124(16): 3065-76.

Dunwoodie, S. L., Clements, M., Sparrow, D. B., Sa, X., Conlon, R. A. and Beddington, R. S. (2002) 'Axial skeletal defects caused by mutation in the spondylocostal dysplasia/pudgy gene Dll3 are associated with disruption of the segmentation clock within the presomitic mesoderm', *Development* 129(7): 1795-806.

Eloy-Trinquet, S. and Nicolas, J. F. (2002a) 'Cell coherence during production of the presomitic mesoderm and somitogenesis in the mouse embryo', *Development* 129(15): 3609-3619.

Eloy-Trinquet, S. and Nicolas, J. F. (2002b) 'Clonal separation and regionalisation during formation of the medial and lateral myotomes in the mouse embryo', *Development* 129(1): 111-122.

Endoh-Yamagami, S., Evangelista, M., Wilson, D., Wen, X., Theunissen, J. W., Phamluong, K., Davis, M., Scales, S. J., Solloway, M. J., de Sauvage, F. J. et al. (2009) 'The mammalian Cos2 homolog Kif7 plays an essential role in modulating Hh signal transduction during development', *Curr Biol* 19(15): 1320-6.

Epstein, D. J., McMahon, A. P. and Joyner, A. L. (1999) 'Regionalization of Sonic hedgehog transcription along the anteroposterior axis of the mouse central nervous system is regulated by Hnf3-dependent and -independent mechanisms', *Development* 126(2): 281-92.

Ericson, J., Morton, S., Kawakami, A., Roelink, H. and Jessell, T. M. (1996) 'Two critical periods of Sonic Hedgehog signaling required for the specification of motor neuron identity', *Cell* 87(4): 661-73.

Ericson, J., Rashbass, P., Schedl, A., Brenner-Morton, S., Kawakami, A., van Heyningen, V., Jessell, T. M. and Briscoe, J. (1997) 'Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling', *Cell* 90(1): 169-80.

Etheridge, L. A., Crawford, T. Q., Zhang, S. and Roelink, H. (2010) 'Evidence for a role of vertebrate Disp1 in long-range Shh signaling', *Development* 137(1): 133-40.

Evrard, Y. A., Lun, Y., Aulehla, A., Gan, L. and Johnson, R. L. (1998) 'lunatic fringe is an essential mediator of somite segmentation and patterning', *Nature* 394(6691): 377-81.

Ferjentsik, Z., Hayashi, S., Dale, J. K., Bessho, Y., Herreman, A., De Strooper, B., del Monte, G., de la Pompa, J. L. and Maroto, M. (2009) 'Notch is a critical component of the mouse somitogenesis oscillator and is essential for the formation of the somites', *PLoS Genet* 5(9): e1000662.

Forsberg, H., Crozet, F. and Brown, N. A. (1998) 'Waves of mouse Lunatic fringe expression, in four-hour cycles at two-hour intervals, precede somite boundary formation', *Curr Biol* 8(18): 1027-30.

Freitas, C., Rodrigues, S., Charrier, J. B., Teillet, M. A. and Palmeirim, I. (2001) 'Evidence for medial/lateral specification and positional information within the presomitic mesoderm', *Development* 128(24): 5139-47.

Galceran, J., Sustmann, C., Hsu, S. C., Folberth, S. and Grosschedl, R. (2004) 'LEF1-mediated regulation of Delta-like1 links Wnt and Notch signaling in somitogenesis', *Genes Dev* 18(22): 2718-23.

Garcia-Martinez, V., Darnell, D. K., Lopez-Sanchez, C., Sosic, D., Olson, E. N. and Schoenwolf, G. C. (1997) 'State of commitment of prospective neural plate and prospective mesoderm in late gastrula/early neurula stages of avian embryos', *Dev Biol* 181(1): 102-15.

Garcia-Martinez, V. and Schoenwolf, G. C. (1992) 'Positional control of mesoderm movement and fate during avian gastrulation and neurulation', *Dev Dyn* 193(3): 249-56.

George, E. L., Georges-Labouesse, E. N., Patel-King, R. S., Rayburn, H. and Hynes, R. O. (1993) 'Defects in mesoderm, neural tube and vascular development in mouse embryos lacking fibronectin', *Development* 119(4): 1079-91.

Georges-Labouesse, E. N., George, E. L., Rayburn, H. and Hynes, R. O. (1996) 'Mesodermal development in mouse embryos mutant for fibronectin', *Dev Dyn* 207(2): 145-56.

Giampietro, P. F., Dunwoodie, S. L., Kusumi, K., Pourquie, O., Tassy, O., Offiah, A. C., Cornier, A. S., Alman, B. A., Blank, R. D., Raggio, C. L. et al. (2009) 'Progress in the understanding of the genetic etiology of vertebral segmentation disorders in humans', *Ann N Y Acad Sci* 1151: 38-67.

Gibb, S., Zagorska, A., Melton, K., Tenin, G., Vacca, I., Trainor, P., Maroto, M. and Dale, K. (2009) 'Interfering with Wnt signalling alters the periodicity of the segmentation clock', *Developmental Biology* 330(1): 21-31.

Goetz, J. A., Singh, S., Suber, L. M., Kull, F. J. and Robbins, D. J. (2006) 'A highly conserved amino-terminal region of sonic hedgehog is required for the formation of its freely diffusible multimeric form', *J Biol Chem* 281(7): 4087-93.

Goetz, S. C. and Anderson, K. V. (2010) 'The primary cilium: a signalling centre during vertebrate development', *Nat Rev Genet* 11(5): 331-44.

Goodrich, L. V., Milenkovic, L., Higgins, K. M. and Scott, M. P. (1997) 'Altered neural cell fates and medulloblastoma in mouse patched mutants', *SCIENCE* 277(5329): 1109-13.

Greco, T. L., Takada, S., Newhouse, M. M., McMahon, J. A., McMahon, A. P. and Camper, S. A. (1996) 'Analysis of the vestigial tail mutation demonstrates that Wnt-3a gene dosage regulates mouse axial development', *Genes Dev* 10(3): 313-24.

Haenig, B. and Kispert, A. (2004) 'Analysis of TBX18 expression in chick embryos', *Dev Genes Evol* 214(8): 407-11.

Hamburger, V. and Hamilton, H. L. (1992) 'A series of normal stages in the development of the chick embryo. 1951', *Dev.Dyn.* 195(4): 231-272.

Hamburguer and Hamilton (1951) 'Stades du développement de l'embryon de poulet', *J.Morph.* 88(1).

Han, C., Belenkaya, T. Y., Wang, B. and Lin, X. (2004) 'Drosophila glypicans control the cell-to-cell movement of Hedgehog by a dynamin-independent process', *Development* 131(3): 601-11.

Hatada, Y. and Stern, C. D. (1994) 'A fate map of the epiblast of the early chick embryo', *Development* 120(10): 2879-89.

Hayashi, S., Shimoda, T., Nakajima, M., Tsukada, Y., Sakumura, Y., Dale, J. K., Maroto, M., Kohno, K., Matsui, T. and Bessho, Y. (2009) 'Sprouty4, an FGF inhibitor, displays cyclic gene expression under the control of the notch segmentation clock in the mouse PSM', *PLoS One* 4(5): e5603.

Haycraft, C. J., Banizs, B., Aydin-Son, Y., Zhang, Q., Michaud, E. J. and Yoder, B. K. (2005) 'Gli2 and Gli3 localize to cilia and require the intraflagellar transport protein polaris for processing and function', *PLoS Genet* 1(4): e53.

Hayward, P., Kalmar, T. and Arias, A. M. (2008) 'Wnt/Notch signalling and information processing during development', *Development* 135(3): 411-24.

Herrgen, L., Ares, S., Morelli, L. G., Schroter, C., Julicher, F. and Oates, A. C. (2010) 'Intercellular coupling regulates the period of the segmentation clock', *Curr Biol* 20(14): 1244-53.

Hirata, H., Bessho, Y., Kokubu, H., Masamizu, Y., Yamada, S., Lewis, J. and Kageyama, R. (2004) 'Instability of Hes7 protein is crucial for the somite segmentation clock', *Nat Genet* 36(7): 750-4.

Hirata, H., Yoshiura, S., Ohtsuka, T., Bessho, Y., Harada, T., Yoshikawa, K. and Kageyama, R. (2002) 'Oscillatory expression of the bHLH factor Hes1 regulated by a negative feedback loop', *Science* 298(5594): 840-843.

Hirsinger, E., Duprez, D., Jouve, C., Malapert, P., Cooke, J. and Pourquie, O. (1997) 'Noggin acts downstream of Wnt and Sonic Hedgehog to antagonize BMP4 in avian somite patterning', *Development* 124(22): 4605-14.

Hofmann, M., Schuster-Gossler, K., Watabe-Rudolph, M., Aulehla, A., Herrmann, B. G. and Gossler, A. (2004) 'WNT signaling, in synergy with T/TBX6, controls Notch signaling by regulating Dll1 expression in the presomitic mesoderm of mouse embryos', *Genes Dev* 18(22): 2712-7.

Holley, S. A. (2007) 'The genetics and embryology of zebrafish metamerism', *Dev Dyn* 236(6): 1422-49.

Holley, S. A., Geisler, R. and Nusslein-Volhard, C. (2000) 'Control of her1 expression during zebrafish somitogenesis by a delta-dependent oscillator and an independent wave-front activity', *Genes Dev* 14(13): 1678-90.

Hooper, J. E. (2003) 'Smoothened translates Hedgehog levels into distinct responses', *Development* 130(17): 3951-63.

Horikawa, K., Ishimatsu, K., Yoshimoto, E., Kondo, S. and Takeda, H. (2006) 'Noise-resistant and synchronized oscillation of the segmentation clock', *Nature* 441(7094): 719-23.

Huangfu, D. and Anderson, K. V. (2006) 'Signaling from Smo to Ci/Gli: conservation and divergence of Hedgehog pathways from Drosophila to vertebrates', *Development* 133(1): 3-14.

Iimura, T. and Pourquie, O. (2006) 'Collinear activation of Hoxb genes during gastrulation is linked to mesoderm cell ingression', *Nature* 442(7102): 568-71.

Iimura, T., Yang, X., Weijer, C. J. and Pourquie, O. (2007) 'Dual mode of paraxial mesoderm formation during chick gastrulation', *Proc Natl Acad Sci U S A* 104(8): 2744-9.

Incardona, J. P., Gaffield, W., Kapur, R. P. and Roelink, H. (1998) 'The teratogenic Veratrum alkaloid cyclopamine inhibits sonic hedgehog signal transduction', *Development* 125(18): 3553-62.

Incardona, J. P., Lee, J. H., Robertson, C. P., Enga, K., Kapur, R. P. and Roelink, H. (2000) 'Receptor-mediated endocytosis of soluble and membrane-tethered Sonic hedgehog by Patched-1', *Proc Natl Acad Sci U S A* 97(22): 12044-9.

Ingham, P. W. and McMahon, A. P. (2001) 'Hedgehog signaling in animal development: paradigms and principles', *Genes Dev* 15: 3059-3087.

Ishikawa, A., Kitajima, S., Takahashi, Y., Kokubo, H., Kanno, J., Inoue, T. and Saga, Y. (2004) 'Mouse Nkd1, a Wnt antagonist, exhibits oscillatory gene expression in the PSM under the control of Notch signaling', *Mech Dev* 121(12): 1443-53.

Jacob, J. and Briscoe, J. (2003) 'Gli proteins and the control of spinal-cord patterning', *EMBO Rep* 4(8): 761-5.

Jenkins, D. (2009) 'Hedgehog signalling: emerging evidence for non-canonical pathways', *Cell Signal* 21(7): 1023-34.

Jeong, J. and McMahon, A. P. (2005) 'Growth and pattern of the mammalian neural tube are governed by partially overlapping feedback activities of the hedgehog antagonists patched 1 and Hhip1', *Development* 132(1): 143-54.

Jiang, Y. J., Aerne, B. L., Smithers, L., Haddon, C., Ish-Horowicz, D. and Lewis, J. (2000) 'Notch signalling and the synchronization of the somite segmentation clock', *Nature* 408(6811): 475-9.

Jouve, C., Iimura, T. and Pourquie, O. (2002) 'Onset of the segmentation clock in the chick embryo: evidence for oscillations in the somite precursors in the primitive streak', *Development* 129(5): 1107-1117.

Jouve, C., Palmeirim, I., Henrique, D., Beckers, J., Gossler, A., Ish-Horowicz, D. and Pourquie, O. (2000) 'Notch signalling is required for cyclic expression of the hairy-like gene HES1 in the presomitic mesoderm', *Development* 127(7): 1421-1429.

Kageyama, R., Ohtsuka, T. and Kobayashi, T. (2007) 'The Hes gene family: repressors and oscillators that orchestrate embryogenesis', *Development* 134(7): 1243-51.

Kawakami, T., Kawcak, T., Li, Y. J., Zhang, W., Hu, Y. and Chuang, P. T. (2002) 'Mouse dispatched mutants fail to distribute hedgehog proteins and are defective in hedgehog signaling', *Development* 129(24): 5753-65.

Kawamura, A., Koshida, S., Hijikata, H., Sakaguchi, T., Kondoh, H. and Takada, S. (2005) 'Zebrafish hairy/enhancer of split protein links FGF signaling to cyclic gene expression in the periodic segmentation of somites', *Genes Dev* 19(10): 1156-61.

Kim, J., Kato, M. and Beachy, P. A. (2009) 'Gli2 trafficking links Hedgehog-dependent activation of Smoothened in the primary cilium to transcriptional activation in the nucleus', *Proc Natl Acad Sci U S A* 106(51): 21666-71.

Kim, P. C., Mo, R. and Hui Cc, C. (2001) 'Murine models of VACTERL syndrome: Role of sonic hedgehog signaling pathway', *J Pediatr Surg* 36(2): 381-4.

Kobayashi, T., Mizuno, H., Imayoshi, I., Furusawa, C., Shirahige, K. and Kageyama, R. (2009) 'The cyclic gene Hes1 contributes to diverse differentiation responses of embryonic stem cells', *Genes Dev* 23(16): 1870-5.

Kraus, F., Haenig, B. and Kispert, A. (2001) 'Cloning and expression analysis of the mouse T-box gene Tbx18', *Mech Dev* 100(1): 83-6.

Kulesa, P. M. and Fraser, S. E. (2002) 'Cell dynamics during somite boundary formation revealed by time-lapse analysis', *SCIENCE* 298(5595): 991-995.

Lee, J., Platt, K. A., Censullo, P. and Ruiz i Altaba, A. (1997) 'Gli1 is a target of Sonic hedgehog that induces ventral neural tube development', *Development* 124(13): 2537-52.

Lei, Q., Zelman, A. K., Kuang, E., Li, S. and Matise, M. P. (2004) 'Transduction of graded Hedgehog signaling by a combination of Gli2 and Gli3 activator functions in the developing spinal cord', *Development* 131(15): 3593-604.

Leimeister, C., Dale, K., Fischer, A., Klamt, B., Hrabe, d. A., Radtke, F., McGrew, M. J., Pourquie, O. and Gessler, M. (2000) 'Oscillating expression of c-Hey2 in the presomitic mesoderm suggests that the segmentation clock may use combinatorial signaling through multiple interacting bHLH factors', *Dev.Biol.* 227(1): 91-103.

Lewis, J., Hanisch, A. and Holder, M. (2009) 'Notch signaling, the segmentation clock, and the patterning of vertebrate somites', *J Biol* 8(4): 44.

Lewis, P. M., Dunn, M. P., McMahon, J. A., Logan, M., Martin, J. F., St-Jacques, B. and McMahon, A. P. (2001) 'Cholesterol modification of sonic hedgehog is required for long-range signaling activity and effective modulation of signaling by Ptc1', *Cell* 105(5): 599-612.

Liem, K. F., Jr., He, M., Ocbina, P. J. and Anderson, K. V. (2009) 'Mouse Kif7/Costal2 is a cilia-associated protein that regulates Sonic hedgehog signaling', *Proc Natl Acad Sci U S A* 106(32): 13377-82.

Ma, Y., Erkner, A., Gong, R., Yao, S., Taipale, J., Basler, K. and Beachy, P. A. (2002) 'Hedgehog-mediated patterning of the mammalian embryo requires transporter-like function of dispatched', *Cell* 111(1): 63-75.

Mallo, M., Vinagre, T. and Carapuco, M. (2009) 'The road to the vertebral formula', *Int J Dev Biol* 53(8-10): 1469-81.

Mansouri, A., Yokota, Y., Wehr, R., Copeland, N. G., Jenkins, N. A. and Gruss, P. (1997) 'Paired-related murine homeobox gene expressed in the developing sclerotome, kidney, and nervous system', *Dev Dyn* 210(1): 53-65.

Marigo, V., Davey, R. A., Zuo, Y., Cunningham, J. M. and Tabin, C. J. (1996a) 'Biochemical evidence that patched is the Hedgehog receptor', *Nature* 384(6605): 176-9.

Marigo, V., Johnson, R. L., Vortkamp, A. and Tabin, C. J. (1996b) 'Sonic hedgehog differentially regulates expression of GLI and GLI3 during limb development', *Dev Biol* 180(1): 273-83.

Maroto, M., Dale, J. K., Dequéant, M. L., Petit, A. C. and Pourquie, O. (2005) 'Synchronised cycling gene oscillations in presomitic mesoderm cells require cell-cell contact', *Int J Dev Biol* 49(2-3): 309-15.

Maroto, M., Reshef, R., Munsterberg, A. E., Koester, S., Goulding, M. and Lassar, A. B. (1997) 'Ectopic Pax-3 activates MyoD and Myf-5 expression in embryonic mesoderm and neural tissue', *Cell* 89(1): 139-48.

Martins, G. G., Rifes, P., Amandio, R., Rodrigues, G., Palmeirim, I. and Thorsteinsdottir, S. (2009) 'Dynamic 3D cell rearrangements guided by a fibronectin matrix underlie somitogenesis', *PLoS One* 4(10): e7429.

Masamizu, Y., Ohtsuka, T., Takashima, Y., Nagahara, H., Takenaka, Y., Yoshikawa, K., Okamura, H. and Kageyama, R. (2006) 'Real-time imaging of the somite segmentation clock: Revelation of unstable oscillators in the individual presomitic mesoderm cells', *Proc.Natl.Acad.Sci.U.S.A* 103(5): 1313-1318.

Matise, M. P., Epstein, D. J., Park, H. L., Platt, K. A. and Joyner, A. L. (1998) 'Gli2 is required for induction of floor plate and adjacent cells, but not most ventral neurons in the mouse central nervous system', *Development* 125(15): 2759-70.

Meier, S. (1979) 'Development of the chick embryo mesoblast. Formation of the embryonic axis and establishment of the metameric pattern', *Dev Biol* 73(1): 24-45.

McGrew, M. J., Dale, J. K., Fraboulet, S. and Pourquie, O. (1998) 'The lunatic fringe gene is a target of the molecular clock linked to somite segmentation in avian embryos', *Curr.Biol.* 8(17): 979-982.

McInerney, D., Schnell, S., Baker, R. E. and Maini, P. K. (2004) 'A mathematical formulation for the cell-cycle model in somitogenesis: analysis, parameter constraints and numerical solutions', *Math Med Biol* 21(2): 85-113.

Meier, S. and Jacobson, A. G. (1982) 'Experimental studies of the origin and expression of metameric pattern in the chick embryo', *J Exp Zool* 219(2): 217-32.

Meinhardt, H. (1986) 'Models of segmentation', *New York: Plenum Press*.

Momiji, H. and Monk, N. A. (2008) 'Dissecting the dynamics of the Hes1 genetic oscillator', *J Theor Biol* 254(4): 784-98.

Morales, A. V., Yasuda, Y. and Ish-Horowicz, D. (2002) 'Periodic Lunatic fringe expression is controlled during segmentation by a cyclic transcriptional enhancer responsive to notch signaling', *Dev Cell* 3(1): 63-74.

Morimoto, M., Takahashi, Y., Endo, M. and Saga, Y. (2005) 'The Mesp2 transcription factor establishes segmental borders by suppressing Notch activity', *Nature* 435(7040): 354-359.

Nakayama, K., Satoh, T., Igari, A., Kageyama, R. and Nishida, E. (2008) 'FGF induces oscillations of Hes1 expression and Ras/ERK activation', *Curr Biol* 18(8): R332-4.

Nicolas, J. F., Mathis, L., Bonnerot, C. and Saurin, W. (1996) 'Evidence in the mouse for self-renewing stem cells in the formation of a segmented longitudinal structure, the myotome', *Development* 122(9): 2933-2946.

Niwa, Y., Masamizu, Y., Liu, T., Nakayama, R., Deng, C. X. and Kageyama, R. (2007) 'The initiation and propagation of Hes7 oscillation are cooperatively regulated by Fgf and notch signaling in the somite segmentation clock', *Dev Cell* 13(2): 298-304.

Oates, A. C. and Ho, R. K. (2002) 'Hairy/E(spl)-related (Her) genes are central components of the segmentation oscillator and display redundancy with the Delta/Notch signaling pathway in the formation of anterior segmental boundaries in the zebrafish', *Development* 129(12): 2929-46.

Olivera-Martinez, I., Coltey, M., Dhouailly, D. and Pourquie, O. (2000) 'Mediolateral somitic origin of ribs and dermis determined by quail-chick chimeras', *Development* 127(21): 4611-7.

Olivera-Martinez, I. and Storey, K. G. (2007) 'Wnt signals provide a timing mechanism for the FGF-retinoid differentiation switch during vertebrate body axis extension', *Development* 134(11): 2125-35.

Ordahl, C. P. and Le Douarin, N. M. (1992) 'Two myogenic lineages within the developing somite', *Development* 114(2): 339-53.

Packard, D. S. (1980) 'Somitogenesis in Cultured Embryos of the Japanese Quail, Coturnix coturnix japonica', *The American Journal of Anatomy* 158: 83-91.

Packard, D. S. and Jacobson, A. G. (1976) 'The Influence of Axial Structures on Chick Somite Formation', *Dev.Biol.* 3: 36-48.

Packard, D. S., Jr. (1978) 'Chick somite determination: the role of factors in young somites and the segmental plate', *J Exp Zool* 203(2): 295-306.

Packard, D. S., Jr. and Meier, S. (1983) 'An experimental study of the somitomeric organization of the avian segmental plate', *Dev Biol* 97(1): 191-202.

Palmeirim, I., Dubrulle, J., Henrique, D., Ish-Horowicz, D. and Pourquie, O. (1998) 'Uncoupling segmentation and somitogenesis in the chick presomitic mesoderm', *Dev.Genet.* 23(1): 77-85.

Palmeirim, I., Henrique, D., Ish-Horowicz, D. and Pourquie, O. (1997) 'Avian hairy gene expression identifies a molecular clock linked to vertebrate segmentation and somitogenesis', *Cell* 91(5): 639-648.

Pascoal, S., Carvalho, C. R., Rodriguez-Leon, J., Delfini, M. C., Duprez, D., Thorsteinsdottir, S. and Palmeirim, I. (2007) 'A Molecular Clock Operates During Chick Autopod Proximal-distal Outgrowth', *J Mol Biol*.

Pearse, R. V., Vogan, K. J. and Tabin, C. J. (2001) 'Ptc1 and Ptc2 Transcripts Provide Distinct Readouts of Hedgehog Signaling Activity during Chick Embryogenesis', *Dev.Biol.* 239: 15-29.

Persson, M., Stamataki, D., te Welscher, P., Andersson, E., Bose, J., Ruther, U., Ericson, J. and Briscoe, J. (2002) 'Dorsal-ventral patterning of the spinal cord requires Gli3 transcriptional repressor activity', *Genes Dev* 16(22): 2865-78.

Pourquie, O., Coltey, M., Breant, C. and Le Douarin, N. M. (1995) 'Control of somite patterning by signals from the lateral plate', *Proc Natl Acad Sci U S A* 92(8): 3219-23.

Pourquie, O., Fan, C. M., Coltey, M., Hirsinger, E., Watanabe, Y., Breant, C., Francis-West, P., Brickell, P., Tessier-Lavigne, M. and Le Douarin, N. M. (1996) 'Lateral and axial signals involved in avian somite patterning: a role for BMP4', *Cell* 84(3): 461-71.

Pourquie, O. and Tam, P. P. L. (2001) 'A nomenclature for prospective somites and phases of cyclic gene expression in the presomitic mesoderm', *Dev Cell* 1(5): 619-20.

Primmett, D. R., Norris, W. E., Carlson, G. J., Keynes, R. J. and Stern, C. D. (1989) 'Periodic segmental anomalies induced by heat shock in the chick embryo are associated with the cell cycle', *Development* 105(1): 119-130.

Primmett, D. R., Stern, C. D. and Keynes, R. J. (1988) 'Heat shock causes repeated segmental anomalies in the chick embryo', *Development* 104(2): 331-9.

Psychoyos, D. and Stern, C. D. (1996) 'Fates and migratory routes of primitive streak cells in the chick embryo', *Development* 122(5): 1523-34.

Reshef, R., Maroto, M. and Lassar, A. B. (1998) 'Regulation of dorsal somitic cell fates: BMPs and Noggin control the timing and pattern of myogenic regulator expression', *Genes Dev* 12(3): 290-303.

Rhee, J., Takahashi, Y., Saga, Y., Wilson-Rawls, J. and Rawls, A. (2003) 'The protocadherin papc is involved in the organization of the epithelium along the segmental border during mouse somitogenesis', *Dev Biol* 254(2): 248-61.

Ribes, V., Balaskas, N., Sasai, N., Cruz, C., Dessaud, E., Cayuso, J., Tozer, S., Yang, L. L., Novitch, B., Marti, E. et al. (2010) 'Distinct Sonic Hedgehog signaling dynamics specify floor plate and ventral neuronal progenitors in the vertebrate neural tube', *Genes Dev* 24(11): 1186-200.

Ribes, V. and Briscoe, J. (2009) 'Establishing and interpreting graded Sonic Hedgehog signaling during vertebrate neural tube patterning: the role of negative feedback', *Cold Spring Harb Perspect Biol* 1(2): a002014.

Ribes, V., Le Roux, I., Rhinn, M., Schuhbaur, B. and Dolle, P. (2009) 'Early mouse caudal development relies on crosstalk between retinoic acid, Shh and Fgf signalling pathways', *Development* 136(4): 665-76.

Ribes, V., Wang, Z., Dolle, P. and Niederreither, K. (2006) 'Retinaldehyde dehydrogenase 2 (RALDH2)-mediated retinoic acid synthesis regulates early mouse embryonic forebrain development by controlling FGF and sonic hedgehog signaling', *Development* 133(2): 351-61.

Rida, P. C., Le Minh, N. and Jiang, Y. J. (2004) 'A Notch feeling of somite segmentation and beyond', *Dev Biol* 265(1): 2-22.

Riddle, R. D., Johnson, R. L., Laufer, E. and Tabin, C. (1993) 'Sonic hedgehog mediates the polarizing activity of the ZPA', *Cell* 75(7): 1401-16.

Riedel-Kruse, I. H., Muller, C. and Oates, A. C. (2007) 'Synchrony dynamics during initiation, failure, and rescue of the segmentation clock', *SCIENCE* 317(5846): 1911-5.

Rifes, P., Carvalho, L., Lopes, C., Andrade, R. P., Rodrigues, G., Palmeirim, I. and Thorsteinsdottir, S. (2007) 'Redefining the role of ectoderm in somitogenesis: a player in the formation of the fibronectin matrix of presomitic mesoderm', *Development* 134(17): 3155-65.

Rohatgi, R., Milenkovic, L. and Scott, M. P. (2007) 'Patched1 regulates hedgehog signaling at the primary cilium', *SCIENCE* 317(5836): 372-6.

Rong, P. M., Teillet, M.-A., Ziller, C. and Le Douarin, N. M. (1992) 'The neural tube/notochord complex is necessary for vertebral but not limb and body wall striated muscle differentiation', *Development* 115: 657-672.

Roy, M. N., Prince, V. E. and Ho, R. K. (1999) 'Heat shock produces periodic somitic disturbances in the zebrafish embryo', *Mech Dev* 85(1-2): 27-34.

Rubin, J. B., Choi, Y. and Segal, R. A. (2002) 'Cerebellar proteoglycans regulate sonic hedgehog responses during development', *Development* 129(9): 2223-32.

Ruiz i Altaba, A. (1999) 'Gli proteins encode context-dependent positive and negative functions: implications for development and disease', *Development* 126: 3205-3216.

Ruiz i Altaba, A., Mas, C. and Stecca, B. (2007) 'The Gli code: an information nexus regulating cell fate, stemness and cancer', *Trends Cell Biol* 17(9): 438-47.

Ruiz i Altaba, A., Sanchez, P. and Dahmane, N. (2002) 'Gli and hedgehog in cancer: tumours, embryos and stem cells', *Nat Rev Cancer* 2(5): 361-72.

Saga, Y. (2007) 'Segmental border is defined by the key transcription factor Mesp2, by means of the suppression of Notch activity', *Dev Dyn* 236(6): 1450-5.

Saga, Y., Hata, N., Koseki, H. and Taketo, M. M. (1997) 'Mesp2: a novel mouse gene expressed in the presegmented mesoderm and essential for segmentation initiation', *Genes Dev* 11(14): 1827-39.

Saha, K. and Schaffer, D. V. (2006) 'Signal dynamics in Sonic hedgehog tissue patterning', *Development* 133(5): 889-900.

Sasai, Y., Kageyama, R., Tagawa, Y., Shigemoto, R. and Nakanishi, S. (1992) 'Two mammalian helix-loop-helix factors structurally related to Drosophila hairy and Enhancer of split', *Genes Dev* 6(12B): 2620-34.

Sawada, A., Shinya, M., Jiang, Y. J., Kawakami, A., Kuroiwa, A. and Takeda, H. (2001) 'Fgf/MAPK signalling is a crucial positional cue in somite boundary formation', *Development* 128(23): 4873-80.

Sawada, K. and Aoyama, H. (1999) 'Fate maps of the primitive streak in chick and quail embryo: ingression timing of progenitor cells of each rostro-caudal axial level of somites', *Int.J Dev.Biol.* 43(8): 809-815.

Schoenwolf, G. C. (1978) 'Effects of complete tail bud extirpation on early development of the posterior region of the chick embryo', *Anat Rec* 192(2): 289-95.

Schoenwolf, G. C., Chandler, N. B. and Smith, J. L. (1985) 'Analysis of the origins and early fates of neural crest cells in caudal regions of avian embryos', *Dev Biol* 110(2): 467-79.

Schoenwolf, G. C., Garcia-Martinez, V. and Dias, M. S. (1992) 'Mesoderm movement and fate during avian gastrulation and neurulation', *Dev Dyn* 193(3): 235-48.

Schragle, J., Huang, R., Christ, B. and Prols, F. (2004) 'Control of the temporal and spatial Uncx4.1 expression in the paraxial mesoderm of avian embryos', *Anat.Embryol.(Berl)* 208(4): 323-332.

Seeley, E. S. and Nachury, M. V. (2010) 'The perennial organelle: assembly and disassembly of the primary cilium', *J Cell Sci* 123(Pt 4): 511-8.

Selleck, M. A. and Stern, C. D. (1991) 'Fate mapping and cell lineage analysis of Hensen's node in the chick embryo', *Development* 112(2): 615-26.

Serth, K., Schuster-Gossler, K., Cordes, R. and Gossler, A. (2003) 'Transcriptional oscillation of lunatic fringe is essential for somitogenesis', *Genes Dev* 17(7): 912-25.

Sewell, W., Sparrow, D. B., Smith, A. J., Gonzalez, D. M., Rappaport, E. F., Dunwoodie, S. L. and Kusumi, K. (2009) 'Cyclical expression of the Notch/Wnt regulator Nrarp requires modulation by Dll3 in somitogenesis', *Dev Biol* 329(2): 400-9.

Shimojo, H., Ohtsuka, T. and Kageyama, R. (2008) 'Oscillations in notch signaling regulate maintenance of neural progenitors', *Neuron* 58(1): 52-64.

Simpson, F., Kerr, M. C. and Wicking, C. (2009) 'Trafficking, development and hedgehog', *Mech Dev* 126(5-6): 279-88.

Skipper M, editor. 2004. Time for segmentation. Nature milestones in development. Milestone 24. Nat Rev Neurosci 5(Suppl):S18.

Stamataki, D., Ulloa, F., Tsoni, S. V., Mynett, A. and Briscoe, J. (2005) 'A gradient of Gli activity mediates graded Sonic Hedgehog signaling in the neural tube', *Genes Dev* 19(5): 626-41.

Stern, C. D. and Bellairs, R. (1984) 'The roles of node regression and elongation of the area pellucida in the formation of somites in avian embryos', *J Embryol Exp Morphol* 81: 75-92.

Stockdale, F. E., Nikovits, W., Jr. and Christ, B. (2000) 'Molecular and cellular biology of avian somite development', *Dev Dyn* 219(3): 304-21.

Suriben, R., Fisher, D. A. and Cheyette, B. N. (2006) 'Dact1 presomitic mesoderm expression oscillates in phase with Axin2 in the somitogenesis clock of mice', *Dev Dyn* 235(11): 3177-83.

Tabin, C. and Wolpert, L. (2007) 'Rethinking the proximodistal axis of the vertebrate limb in the molecular era', *Genes Dev* 21(12): 1433-42.

Taipale, J., Cooper, M. K., Maiti, T. and Beachy, P. A. (2002) 'Patched acts catalytically to suppress the activity of Smoothened', *Nature* 418(6900): 892-7.

Takahashi, Y., Koizumi, K., Takagi, A., Kitajima, S., Inoue, T., Koseki, H. and Saga, Y. (2000) 'Mesp2 initiates somite segmentation through the Notch signalling pathway', *Nat Genet* 25(4): 390-6.

Takebayashi, K., Sasai, Y., Sakai, Y., Watanabe, T., Nakanishi, S. and Kageyama, R. (1994) 'Structure, chromosomal locus, and promoter analysis of the gene encoding the mouse helix-loop-helix factor HES-1. Negative autoregulation through the multiple N box elements', *J Biol Chem* 269(7): 5150-6.

Tam, P. P. (1981) 'The control of somitogenesis in mouse embryos', *J Embryol Exp Morphol* 65 Suppl: 103-28.

Tanaka, M. and Tickle, C. (2004) 'Tbx18 and boundary formation in chick somite and wing development', *Dev Biol* 268(2): 470-80.

Teillet, M., Watanabe, Y., Jeffs, P., Duprez, D., Lapointe, F. and Le Douarin, N. M. (1998) 'Sonic hedgehog is required for survival of both myogenic and chondrogenic somitic lineages', *Development* 125(11): 2019-30.

Tenin, G., Wright, D., Ferjentsik, Z., Bone, R., McGrew, M. J. and Maroto, M. (2010) 'The chick somitogenesis oscillator is arrested before all paraxial mesoderm is segmented into somites', *BMC Dev Biol* 10: 24.

Tenzen, T., Allen, B. L., Cole, F., Kang, J. S., Krauss, R. S. and McMahon, A. P. (2006) 'The cell surface membrane proteins Cdo and Boc are components and targets of the Hedgehog signaling pathway and feedback network in mice', *Dev Cell* 10(5): 647-56.

Tian, H., Jeong, J., Harfe, B. D., Tabin, C. J. and McMahon, A. P. (2005) 'Mouse Disp1 is required in sonic hedgehog-expressing cells for paracrine activity of the cholesterol-modified ligand', *Development* 132(1): 133-42.

Turnpenny, P. D., Alman, B., Cornier, A. S., Giampietro, P. F., Offiah, A., Tassy, O., Pourquie, O., Kusumi, K. and Dunwoodie, S. (2007) 'Abnormal vertebral segmentation and the notch signaling pathway in man', *Dev Dyn* 236(6): 1456-74.

Uriu, K., Morishita, Y. and Iwasa, Y. (2010) 'Random cell movement promotes synchronization of the segmentation clock', *Proc Natl Acad Sci U S A* 107(11): 4979-84.

Varjosalo, M. and Taipale, J. (2008) 'Hedgehog: functions and mechanisms', *Genes Dev* 22(18): 2454-72.

Villavicencio, E. H., Walterhouse, D. O. and Iannaccone, P. M. (2000) 'The sonic hedgehog-patched-gli pathway in human development and disease', *Am J Hum Genet* 67(5): 1047-54.

Vyas, N., Goswami, D., Manonmani, A., Sharma, P., Ranganath, H. A., VijayRaghavan, K., Shashidhara, L. S., Sowdhamini, R. and Mayor, S. (2008) 'Nanoscale organization of hedgehog is essential for long-range signaling', *Cell* 133(7): 1214-27.

Wacker, S. A., Jansen, H. J., McNulty, C. L., Houtzager, E. and Durston, A. J. (2004) 'Timed interactions between the Hox expressing non-organiser mesoderm and the Spemann organiser generate positional information during vertebrate gastrulation', *Dev Biol* 268(1): 207-19.

Wahl, M. B., Deng, C., Lewandoski, M. and Pourquie, O. (2007) 'FGF signaling acts upstream of the NOTCH and WNT signaling pathways to control segmentation clock oscillations in mouse somitogenesis', *Development* 134(22): 4033-41.

Wellik, D. M. (2007) 'Hox patterning of the vertebrate axial skeleton', *Dev Dyn* 236(9): 2454-63.

Wijgerde, M., McMahon, J. A., Rule, M. and McMahon, A. P. (2002) 'A direct requirement for Hedgehog signaling for normal specification of all ventral progenitor domains in the presumptive mammalian spinal cord', *Genes Dev* 16(22): 2849-64.

William, D. A., Saitta, B., Gibson, J. D., Traas, J., Markov, V., Gonzalez, D. M., Sewell, W., Anderson, D. M., Pratt, S. C., Rappaport, E. F. et al. (2007) 'Identification of oscillatory genes in somitogenesis from functional genomic analysis of a human mesenchymal stem cell model', *Dev Biol* 305(1): 172-86.

Wolpert, L. (1969) 'Positional information and the spatial pattern of cellular differentiation', *J Theor Biol* 25(1): 1-47.

Wright, D., Ferjentsik, Z., Chong, S. W., Qiu, X., Jiang, Y. J., Malapert, P., Pourquie, O., Van Hateren, N., Wilson, S. A., Franco, C. et al. (2009) 'Cyclic Nrarp mRNA expression is regulated by the somitic oscillator but Nrarp protein levels do not oscillate', *Dev Dyn* 238(12): 3043-55.

Yamamoto, A., Kemp, C., Bachiller, D., Geissert, D. and De Robertis, E. M. (2000) 'Mouse paraxial protocadherin is expressed in trunk mesoderm and is not essential for mouse development', *Genesis* 27(2): 49-57.

Yang, J. T., Bader, B. L., Kreidberg, J. A., Ullman-Cullere, M., Trevithick, J. E. and Hynes, R. O. (1999) 'Overlapping and independent functions of fibronectin receptor integrins in early mesodermal development', *Dev Biol* 215(2): 264-77.

Yang, X., Dormann, D., Munsterberg, A. E. and Weijer, C. J. (2002) 'Cell movement patterns during gastrulation in the chick are controlled by positive and negative chemotaxis mediated by FGF4 and FGF8', *Dev Cell* 3(3): 425-37.

Yang, Y., Drossopoulou, G., Chuang, P. T., Duprez, D., Marti, E., Bumcrot, D., Vargesson, N., Clarke, J., Niswander, L., McMahon, A. et al. (1997) 'Relationship between dose, distance and time in Sonic Hedgehog-mediated regulation of anteroposterior polarity in the chick limb', *Development* 124(21): 4393-404.

Yasuhiko, Y., Haraguchi, S., Kitajima, S., Takahashi, Y., Kanno, J. and Saga, Y. (2006) 'Tbx6-mediated Notch signaling controls somite-specific Mesp2 expression', *Proc Natl Acad Sci U S A* 103(10): 3651-6.

Yin, Y., Bangs, F., Paton, I. R., Prescott, A., James, J., Davey, M. G., Whitley, P., Genikhovich, G., Technau, U., Burt, D. W. et al. (2009) 'The Talpid3 gene (KIAA0586) encodes a centrosomal protein that is essential for primary cilia formation', *Development* 136(4): 655-64.

Zhang, N. and Gridley, T. (1998) 'Defects in somite formation in lunatic fringe-deficient mice', *Nature* 394(6691): 374-7.

Zhao, Y., Tong, C. and Jiang, J. (2007) 'Hedgehog regulates smoothened activity by inducing a conformational switch', *Nature* 450(7167): 252-8.

# CHAPTER2. SONIC HEDGEHOG

## IN TEMPORAL CONTROL OF SOMITE FORMATION

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# Sonic hedgehog in temporal control of somite formation

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**Vertebrate embryo somite formation is temporally controlled by the cyclic expression of somitogenesis clock genes in the presomitic mesoderm (PSM). The somitogenesis clock is believed to be an intrinsic property of this tissue, operating independently of embryonic midline structures and the signaling molecules produced therein, namely Sonic hedgehog (Shh). This work revisits the notochord signaling contribution to temporal control of PSM segmentation by assessing the rate and number of somites formed and somitogenesis molecular clock gene expression oscillations upon notochord ablation. The absence of the notochord causes a delay in somite formation, accompanied by an increase in the period of molecular clock oscillations. Shh is the notochord-derived signal responsible for this effect, as these alterations are recapitulated by Shh signaling inhibitors and rescued by an external Shh supply. We have characterized chick *smoothened* expression pattern and have found that the PSM expresses both *patched1* and *smoothened* Shh signal transducers. Upon notochord ablation, *patched1*, *gli1*, and *fgf8* are down-regulated, whereas *gli2* and *gli3* are overexpressed. Strikingly, notochord-deprived PSM segmentation rate recovers over time, concomitant with *raldh2* overexpression. Accordingly, exogenous RA supplement rescues notochord ablation effects on somite formation. A model is presented in which Shh and RA pathways converge to inhibit PSM Gli activity, ensuring timely somite formation. Altogether, our data provide evidence that a balance between different pathways ensures the robustness of timely somite formation and that notochord-derived Shh is a component of the molecular network regulating the pace of the somitogenesis clock.**

somitogenesis | molecular clock | notochord

Vertebrate body segmented organization relies on the progressive formation of early embryonic vertebrae precursor structures—somites—flanking bilaterally the axial organs, notochord, and neural tube. Somitogenesis is a reiterated process occurring over time with strict periodicity. In the trunk of the chicken embryo, a pair of somites is formed every 90 min from the anterior tip of presomitic mesoderm (PSM). As development proceeds, ventral somitic cells migrate around the axial organs, giving rise to segmented structures such as vertebrae, intervertebral disks, and ribs, whereas more dorsal somitic cells give rise to the dermis of the back and all of the striated muscles of the adult body (1). Underlying somitogenesis periodicity is a molecular clock first evidenced by the cyclic expression of *hairy1* in chick PSM cells with 90-min periodicity, corresponding to the time of somite formation (2). Currently, multiple genes belonging to Notch, Wnt, and Fgf signaling pathways are known to have similar oscillatory behavior and to participate in the embryo segmentation clock (1, 3). Posterior PSM cells in an undetermined state are influenced by high FGF/Wnt levels, which fade away anteriorly, counteracted by an opposing gradient of RA (4–6). A determination front is established in the intersection of these opposite gradients at the level of somites –III to –IV, and cells located anteriorly to this front are determined to form somites (6).

Packard et al. described somite formation to be independent of the neural tube and notochord (7, 8) and today this is widely accepted. The somitogenesis clock is also believed to operate independently of these axial structures (2). However, there is an intriguing report showing that when a quail PSM is grafted into a chicken embryo, the grafted tissue progressively adjusts somite formation to that of the host contralateral part (9). This is particularly evident in the somites formed by cells that were located posterior to the donor determination front when the graft was performed, strongly suggesting that undetermined PSM requires a signal coming from the embryo midline structures for subsequent somite formation. Notochord and neural tube floor plate produce Sonic hedgehog (Shh), a morphogen central to growth, patterning, and morphogenesis (10), implicated in somite sclerotome induction (11) and in survival of medial somitic cells (12, 13). These functions have been described to account for the lack of spinal column observed in Shh KO mice (14). However, it is commonly believed that Shh signaling does not play a role in PSM segmentation or in the somitogenesis clock.

In the present work, we evaluated the notochord and Shh signaling contribution to PSM segmentation preceding somite formation. We show that notochord ablation delays somite formation and molecular clock oscillations, and that Shh is the notochord-derived signal regulating these events. Furthermore, our results show that the delay in somite formation in notochord-deprived PSMs is overcome in time, mediated by RA signaling. Altogether, our data identify embryo midline-derived Shh as a component of the molecular network regulating the pace of the somitogenesis clock and reveal a cross-talk with the RA pathway to ensure the robustness of somite formation.

## Results

**Timely Somite Segmentation of Undetermined PSM Tissue Requires Presence of Notochord.** To evaluate the role of the notochord in PSM segmentation, the posterior part of chicken embryos was cut along the embryo midline, generating two embryo explants (Fig. 1A): a control explant containing half of the neural tube, the floor plate, and the notochord ($No^+$), and an experimental explant with half of the neural tube but without the floor plate and notochord ($No^-$). Explant pairs were cultured separately for different time periods (4.5, 7.5, 9, and 12 h), and the number of somites formed in culture was assessed (Fig. 1 *B* and *C*). The

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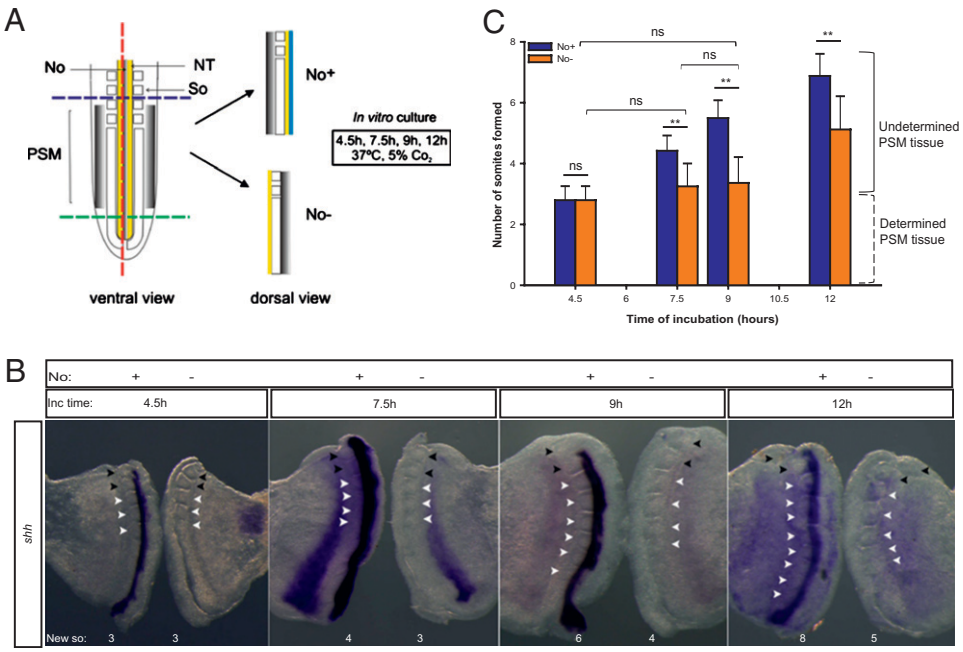


**Fig. 1.** Notochord removal impairs timely somite segmentation of the undetermined PSM in vitro. (*A*) Schematic representation of explant delimitation and excision. No, notochord; NT, neural tube; So, somite; PSM, presomitic mesoderm. (*B*) Evidence for the presence ($No^+$)/absence ($No^-$) of the notochord in control and experimental explants, by in situ hybridization for *shh*. Black arrowheads indicate somites formed before culture; white arrowheads indicate somites formed during incubation (New so). (*C*) Graphic representation of mean number of somites formed de novo upon 4.5, 7.5, 9, or 12 h of incubation. Data are mean ± SD. $^{**}P < 0.05$. ns, not statistically significant.

presence or absence of notochord on the control and experimental explants was randomly confirmed by in situ hybridization using a *sonic hedgehog* (*shh*) antisense RNA probe (Fig. 1*B*).

Explants incubated for 4.5 h formed three somites at the expected rate of one somite/90 min, even in the absence of the notochord ($n = 68$) (Fig. 1 *B* and *C*). Surprisingly, for greater incubation periods of 7.5 h ($n = 33$) and 9 h ($n = 100$), the PSM tissue did not segment to the same extent in the absence of notochord as in control explants (Fig. 1 *B* and *C*). In fact, somite formation considerably slowed down from 4.5 h to 9 h of incubation. Both PSM cell apoptosis and proliferation levels were assessed, and showed that the results obtained were not caused by alterations in either of these cellular processes (Fig. S1). Strikingly, from 9 h to 12 h ($n = 25$), an average of two new somites was formed even in the absence of the notochord, suggesting a recovery of the system (Fig. 1 *B* and *C*). Our experimental approach was reproduced in ovo; upon 9 h ($n = 28$) or 15 h ($n = 24$) of reincubation, the results obtained recapitulate those observed in explant cultures: the side without notochord consistently formed less somites than the control part (Fig. 2 and Movie S1). Moreover, somite formation rate is recovered over time and, accordingly, a difference of two somites in control and experimental sides is maintained after 9, 12, or 15 h of incubation (Figs. 1*C* and 2*C*). In some embryos the size of the slit allowed to observe a delay in somite segmentation in the PSM facing the incision, whereas timely bilateral somite formation occurred caudal to the slit, leaving a rostral gap of unsegmented tissue with approximately two somites length (Fig. 2*D*). Because somite formation periodicity in the absence of the notochord is already reestablished at 15 h (Figs. 1*C* and 2*C*), $No^-$ tissue will undergo further segmentation in a timely fashion, concomitantly with the generation of new somites caudal to the slit. Accordingly, after a 24 h incubation, both control and experimental PSMs are completely segmented indicating that notochord deprivation does not impair, but transiently delays somite formation (Fig. S2 *A* and *B*).

Altogether, we show that the undetermined PSM tissue depends on the presence of the notochord for timely somite segmentation. Moreover, as long as the PSM cells retain contact with the notochord, they preserve autonomous correct temporal information. PSM rostral to the determination front no longer requires the notochord to segment at normal rate.

**Notochord Ablation Alters Clock and Determination Front Gene Expression.** Because notochord ablation affected temporal control of morphological somite formation, we evaluated the expression of genes involved in the somitogenesis clock and determination front establishment. Explants such as those previously described ($No^+$ and $No^-$) were incubated for 4.5 or 9 h and hybridized with probes for the clock genes *hairy2* and *lunatic fringe* (*lfng*), and determination front genes *fgf8* and *raldh2* (Fig. 3). Molecular clock gene expression was significantly altered in the absence of the notochord both after 4.5 h (*lfng*, $n = 4/4$; *hairy2*, $n = 8/11$) and 9 h (*lfng*, $n = 19/22$; *hairy2*, $n = 15/18$) (Fig. 3). The same could be observed upon in ovo microsurgery (Fig. S2*C*). Furthermore, in notochord-deprived explants the expression domains of both *fgf8* (Fig. 3) and its downstream target *sprouty1* (Fig. S3) were shifted caudally. However, this was not the primary cause for somite formation delay, because FGF8 supplementation did not rescue somite formation in $No^-$ explants and inhibition of FGF signaling in $No^+$ explants did not mimic Shh deprivation (Fig. S4). *Raldh2* expression was unaltered by notochord ablation upon 4.5 h of incubation ($n = 9/10$) but was highly overexpressed after 9 h ($n = 4/5$) (Fig. 3). The expression of *meis2*, a RA downstream target, further confirms these results and was already slightly up-regulated after 4.5 h (Fig. S3). As a control, two equivalent $No^+$ explants were generated by splitting the notochord in half, and no alterations in segmentation rate or gene expression were observed (Fig. S5). In conclusion, somite formation delay in the absence of the notochord is accompanied by alterations in both molecular clock and determination front gene expression.

**Ectopic Shh Replaces Notochord in Temporal Control of Gene Expression and Somite Formation.** *Shh* is one of the most well-studied signaling molecules produced by the notochord. Shh-producing (QT6-Shh) or control (QT6-ctrl) QT6 cells were juxtaposed to $No^-$ explants which were cultured for 9 h in parallel to their $No^+$ counterpart (Fig. 4A). $No^-$/QT6-ctrl ($n = 43$) failed to form the expected number of somites and presented gene expression perturbations, similarly to what had been previously observed for the $No^-$

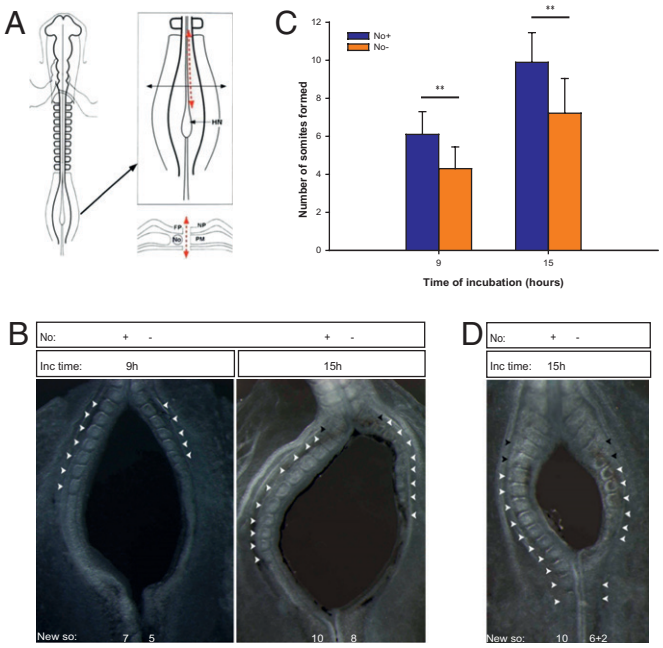


**Fig. 2.** Notochord ablation in ovo delays somite segmentation of the undetermined PSM. (*A*) Schematic representation of embryo microsurgical operation. Only one of the embryo's PSMs (PM) was left in contact with the notochord (No). HN, Hensen's node; FP, floor plate; NP, neural plate. (*B*) Representative results obtained in operated embryos after a reincubation period of 9 or 15 h. (*C*) Graphic representation of the mean number of somites formed after 9 or 15 h. Data are mean ± SD. $^{**}P < 0.001$. (*D*) Operated embryo reincubated for 15 h where a gap in somite formation is observed, resulting from the absence of notochord ($No^-$) in the PSM tissue juxtaposed to the surgical slit, whereas reestablished contact of the posterior PSM with the notochord reinstates timely somite formation. Presence ($No^+$) or absence ($No^-$) of notochord is indicated. Black arrowheads indicate somites formed before the surgical intervention; white arrowheads indicate somites formed during reincubation (New so).

explants (Fig. 4 *B* and *C*). Notably, $No^-$/QT6-Shh explants and $No^+$ controls presented the same number of somites and comparable expression patterns for the molecular clock genes *hairy2* ($n = 9/14$) and *lfng* ($n = 9/12$) and for the determination front genes *fgf8* ($n = 4/6$) and *raldh2* ($n = 3/3$) (Fig. 4 *B* and *C*). Altogether, the results indicate that Shh is able to replace the notochord in control of timely somite segmentation and underlying gene expression dynamics.

**Shh Signaling Controls Clock Pace and Somite Formation.** Shh signaling is transduced by a receptor complex that includes two transmembrane proteins, Patched (Ptch) and Smoothened (Smo). Shh association with Ptch allows Smo activity, whereas in the absence of Shh, Ptch binds and inhibits Smo. Among the many target genes regulated by Shh signaling are *ptch* and *gli* signal transducers (15). To investigate the Shh pathway components operating in somite segmentation, we characterized the expression patterns of *smo* and *ptch1/2* (Fig. S6). We found that notochord-adjacent PSM cells express both *smo* and *ptch1/2*, enabling them to respond to notochord-derived Shh.

We performed chemical inhibition of Shh pathway in $No^+$ explants and the results obtained mimicked the absence of notochord, leading to a similar delay in somite formation (Fig. 5, *A*–*C*). Furthermore, the expression patterns of molecular clock genes are different in control and Shh-inhibited explants (Fig. 5*B*). Shh inhibition, however, does not halt molecular clock oscillations: cyclopamine-treated explants incubated for time periods differing by 30 min (6 h and 6.5 h) presented dissimilar phases of molecular clock gene expression (Fig. 5*D*). In accordance to the observed delay in somite formation, Shh-inhibited explants incubated for time periods differing by 90 min (6 h and 7.5 h) differed in the phases of molecular clock gene expression (Fig. 5*E*). Taken together, these results indicate that, in the absence of Shh signaling, the period of the clock is no longer 90 min.

To evaluate the delay in the molecular clock, we cultured pairs of Shh-inhibited explants for time periods differing by 1 h 10 min, 2 h, 2 h 15 min, or 2.5 h. All explant pairs presented different phases of expression of the clock and the same number of somites. When explants were incubated with a time difference of 2 h 45 min (Fig. 5*F*), 27% of the cases presented similar clock gene expression, but no extra somite formed. Finally, when differing by 3 h, 85% of the explants had formed one extra somite but already presented different molecular clock patterns. These results indicate that clock oscillations are severely delayed in the absence of Shh, with a new time period between 2 h 45 min and 3 h. The imprecision in the determination of the new oscillation period could be due to differing levels of perturbation of the system imposed by Shh deprivation, which can be dependent on the phase of the molecular clock at the moment of notochord ablation. In conclusion, Shh ensures timely somite segmentation by setting the pace of the somitogenesis molecular clock.

**Retinoic Acid Is Able to Recover Somite Formation Rate in the Absence of Notochord.** Notochord-deprived PSMs present delayed somite formation from 4.5 h of incubation onward, accompanied by an overexpression of *gli2* ($n = 4/4$) and *gli3* ($n = 11/11$), a slight up-regulation of *smo* ($n = 5/5$) and down-regulation of both *ptch1* ($n = 5/5$) and *gli1* ($n = 3/3$) (Fig. 6). Timely somite formation is resumed after 9 h and this is not due to a re-establishment of Shh pathway components throughout this time period, as their levels of expression do not revert to basal state (Fig. 6). At 9 h, an intriguing overexpression of *raldh2* is observed that is not present at 4.5 h (Fig. 3). To test whether increased amounts of retinoic acid (RA) could mediate the recovery of somite formation rate, $No^-$ explants were incubated in RA-conditioned medium, and the number of formed somites after 9 h was assessed (Fig. 7 *A* and *B*). Noticeably, a greater number of somites is formed in $No^-$ explants when the medium is supplemented with RA. Moreover, the total number of somites formed in these conditions is not significantly

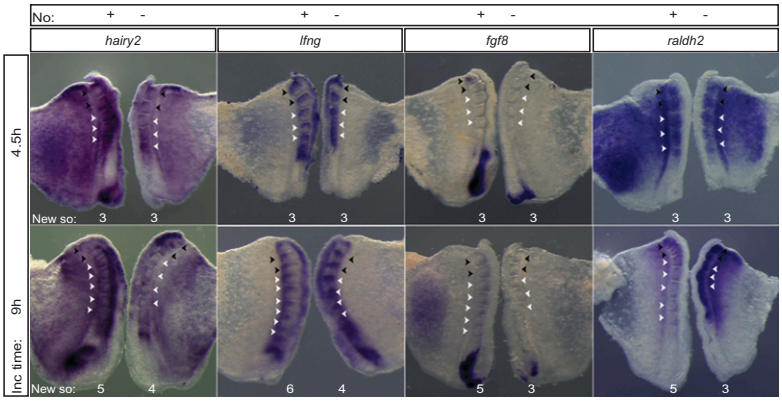


**Fig. 3.** Notochord ablation alters somitogenesis clock and determination front gene expression. Expression patterns of *hairy2, lfng, fgf8,* and *raldh2,* evaluated by in situ hybridization in explants cultured for 4.5 or 9 h. Presence ($No^+$) or absence ($No^-$) of the notochord is indicated. Black arrowheads indicate somites formed before culture; white arrowheads indicate somites formed during incubation (New so).

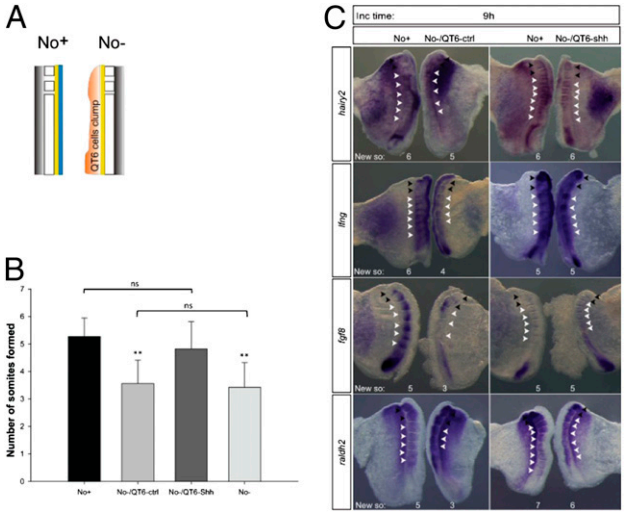


**Fig. 4.** Exogenous Shh supply replaces the notochord in temporal control of somite formation and gene expression. (*A*) Schematic representation of a clump (pink) of control QT6 fibroblasts (QT6-ctrl) or QT6-producing Shh cells (QT6-Shh) juxtaposed to the neural tube (yellow) of explants without notochord (No$^-$). (*B*) Graphic representation of mean number of somites formed by explants with notochord (No$^+$), without notochord (No$^-$), and without notochord but with control (No$^-$/QT6-ctrl) or Shh-producing (No$^-$/QT6-Shh) cells. $^{**}P < 0.05$ vs. No$^+$ or No$^-$/QT6-Shh. ns, not statistically significant. Data are mean ± SD. (*C*) Explants with juxtaposed cells were hybridized for *hairy2, lfng, fgf8,* and *raldh2.* Black arrowheads indicate somites formed before culture; white arrowheads indicate somites formed during incubation (New so).

different from that observed in the No$^+$ explant (Fig. 7*B*). Our results strongly suggest that an increase in RA signaling can mediate the recovery of somite formation in the absence of Shh. However, greatly increasing the amounts of RA, Shh, or both could not accelerate somite formation rate (Fig. S7).

## Discussion

**Somite Segmentation Relies on Axial Structures.** Segmentation of paraxial mesoderm has been classically described as an intrinsic mechanism independent of axial structures (16). PSM tissue incubated for 14 h, with or without the axial structures, formed the same number of somites (7, 8), suggesting that these structures do not contribute to somite formation. However, the rate of PSM segmentation during the incubation period was not evaluated. When the molecular clock genes were described, their expression oscillations were also shown to occur independently of axial structures (2, 17, 18). Nevertheless, these studies were performed only up to 4.5 h of incubation, and we now know that this accounts only for determined PSM segmentation (6). Only one report has suggested that contact with tissues near the midline influences somite formation (9).

We have revisited this issue by evaluating somite formation rate over time in the presence or absence of the notochord. The temporal framework used allowed us to assess segmentation of both determined and undetermined PSM. Our results clearly show that the anterior third of the PSM does not require the notochord for timely segmentation, indicating that this tissue is determined not only spatially (6) but also temporally (this work). Segmentation of the undetermined PSM, however, is significantly delayed in the absence of the notochord. This is particularly evident for the first three undetermined somites, which resume proper segmentation only after 9–12 h of incubation. Hence, although the absence of the notochord does not impair segmentation (this work; 7, 8), we show that the strict, characteristic rate of embryo somite formation is dependent upon a signal from the notochord.

**Shh Produced by Embryo Midline Structures Ensures Somite Formation Rate.** Shh signaling pathway is not classically associated with somite segmentation. In fact, neither the Shh downstream Gli effectors nor the pathway's membrane receptors have been reported to be expressed throughout the PSM (19, 20). We show that *ptch1* is expressed in the medial PSM tissue and that *smo* is ubiquitously present in the PSM, creating the conditions for Shh signaling readout in this tissue.

Embryo midline structures produce diffusible Shh. Our results indicate that the notochord role on somite formation rate may be the production of a Shh signal delivered to the adjacent PSM. In fact, exogenous supply of this protein rescued timely somite formation in the absence of the notochord. Moreover, in the presence of canonical Shh inhibitors somite formation rate was perturbed to the same extent as when the notochord was removed. These experiments strongly suggest that embryo midline structures ensure strict control of somite formation rate through the supply of canonical Shh signal to the PSM.

**Somitogenesis Clock Periodicity Is Modulated by Shh Signaling.** Could a perturbation in the clock period underlie the delay in somite

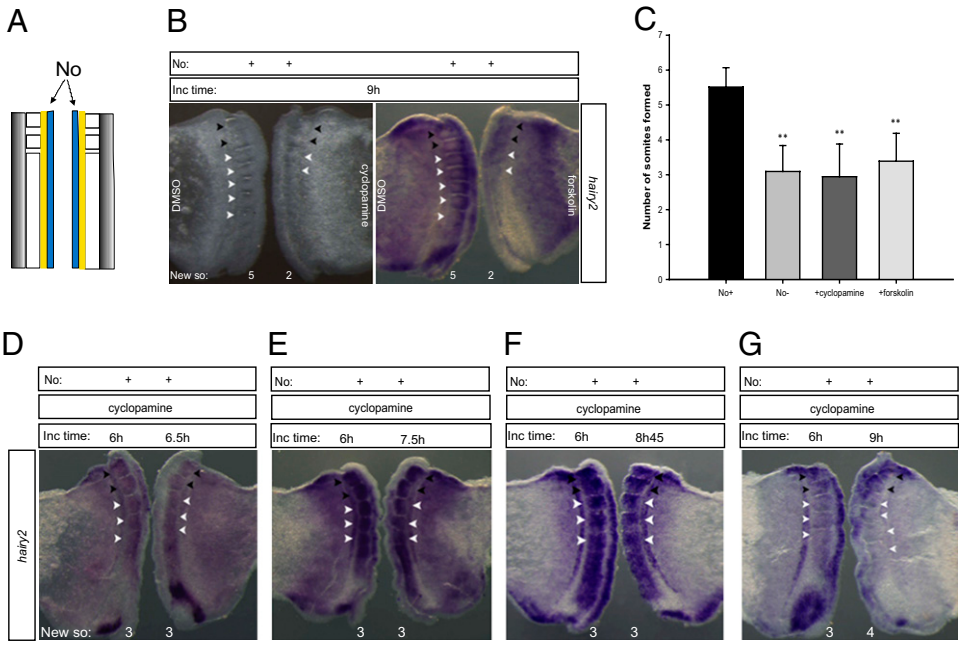


**Fig. 5.** Inhibition of Shh signaling delays the pace of the clock. (*A*) Schematic representation of explant delimitation and excision. (*B*) Bisected-notochord explants were incubated for 9 h with DMSO (control explant) and with either 10 μg/mL cyclopamine or 25 μg/mL forskolin (experimental explant). Forskolin-treated explant and corresponding control were hybridized for *hairy2*. (*C*) Graphic representation of mean number of somites formed by explants with notochord (No$^+$), without notochord (No$^-$), and with notochord but incubated in the presence of cyclopamine or forskolin after 9 h of incubation. $^{**}P < 0.05$ vs. No$^+$. ns, Not statistically significant. Data are mean ± SD. (*D–G*) Explants with bisected notochord treated with cyclopamine were cultured for the indicated time periods and hybridized with *hairy2* probe. Black arrowheads indicate somites formed before culture; white arrowheads indicate somites formed during incubation (New so).

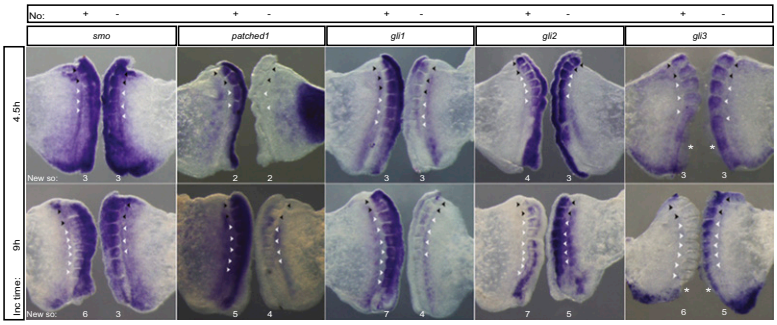


**Fig. 6.** Expression of Shh pathway components in the PSM is altered by notochord ablation. Expression patterns of *smo, ptch1, gli1, gli2,* and *gli3* genes evaluated by in situ hybridization in explants cultured for 4.5 or 9 h. *Neural tube removal from explants for better visualization of PSM *gli3* expression. Presence ($No^+$) or absence ($No^-$) of the notochord is indicated. Black arrowheads indicate somites formed before culture: white arrowheads indicate somites formed during incubation (New so).

formation observed in the absence of Shh? When PSM tissue is deprived of Shh signaling, either by notochord ablation or chemical inhibition, *hairy2*/*lfng* expression patterns are significantly different from those obtained in control conditions. Moreover, an exogenous Shh supply to notochord-ablated explants recovered molecular clock expression patterns. Hence, we could detect a clear correlation between the presence of Shh and both timely somite formation and molecular clock gene oscillations. Our group has previously shown that only the medial-most PSM (M-PSM) cells possess intrinsic information for both somite formation and molecular clock gene expression (21). The study suggested that a signal supplied by M-PSM travels along the medial-lateral PSM axis, ensuring both timely segmentation and cyclic clock expression. However, the nature of this signal was not identified (21). The results obtained throughout the present work suggest that M-PSM cells are exposed to notochord-derived Shh signaling, as evidenced by *ptch1*/*2* restricted expression, providing them crucial information for timely clock oscillations and somite formation. M-PSM cells are then capable of instructing and recruiting their lateral neighbors for segmentation. This hypothesis is supported by the observation that somites formed in the absence of Shh are smaller along the medial-lateral (M-L) axis (Fig. S2B). How this is achieved in vivo is subject of ongoing studies in the laboratory.

Inhibition of Wnt signaling has also been described to perturb the molecular clock (22). We found that *axin2* expression is unaltered in 60% of the notochord-ablated explants (Fig. S8) and the other 40% are only slightly down-regulated after 4.5 h. Because clock expression is perturbed in a much higher percentage of cases (80%), somite formation delay in the absence of Shh should not be due primarily to an impairment of Wnt signaling. Moreover, Wnt does not seem to be involved in the recovery of timely segmentation, as *axin2* is clearly down-regulated after 9 h (Fig. S8).

Somitogenesis clock gene expression perturbation in the absence of Shh signaling was not due to a halt in gene oscillations, but to an alteration of the clock period. In fact, the period of *hairy2* expression cycles is considerably enlarged in the absence of Shh signaling, from 1.5 h to a time point between 2 h 45 min and 3 h. Significantly, somite formation in these conditions is also greatly delayed, reinforcing the dependence of timely somite formation on the pace of clock oscillations. Interestingly, somite formation delay observed upon Shh inhibition was dose dependent, as fewer somites were formed with increased inhibitor concentrations (Fig. S9). In situ hybridization on sections suggests the existence of a *ptch1* expression gradient, with higher levels in the determined PSM (Fig. S6D). It is tempting to postulate that an anterior–posterior gradient of Shh activity could account for the different segmentation rates observed for rostral (early) and caudal (late) somites. Shh knock-out mice present a striking absence of the entire spinal column and most of the ribs (14). Our data suggest that a perturbation in somite segmentation could be contributing to this phenotype.

**Shh and RA Pathways Interplay with the Molecular Clock to Ensure Timely Somite Formation.** This work shows that Shh produced by the notochord/floor plate is required to maintain timely operation of the molecular clock and the correct pace of somite formation (Fig. 7*C, Left*). An important consequence of PSM isolation from its source of Shh is the up-regulation of *gli2* and *gli3* (Fig. 7*C, Middle*), which corroborates previous reports (19). This strongly suggests that low *gli* levels in the PSM are not due to the lack of Shh signaling readout, as is commonly believed, but rather are actively restricted by notochord-derived Shh. Another striking molecular consequence of notochord ablation is the down-regulation of *fgf8* expression in the PSM, showing that Shh signaling is required to preserve the *fgf8* gradient underlying the determination front. These molecular changes, observed in the PSM after 4.5 h of incubation in the absence of Shh, most probably account for the subsequent delay in somite formation rate and molecular clock oscillations.

The PSM *fgf8* gradient counteracts an opposing RA gradient (5). Hence, the reduction of *fgf8* levels in the absence of Shh could allow the observed up-regulation of Raldh2 after 9 h of incubation (Fig. 7*C, Right*). Under these molecular conditions, there is a recovery of somite formation rate. Accordingly, we show that an external RA supply is able to rescue timely somite formation in the absence of Shh (Fig. 7 *A* and *B*). Although RA has been shown to

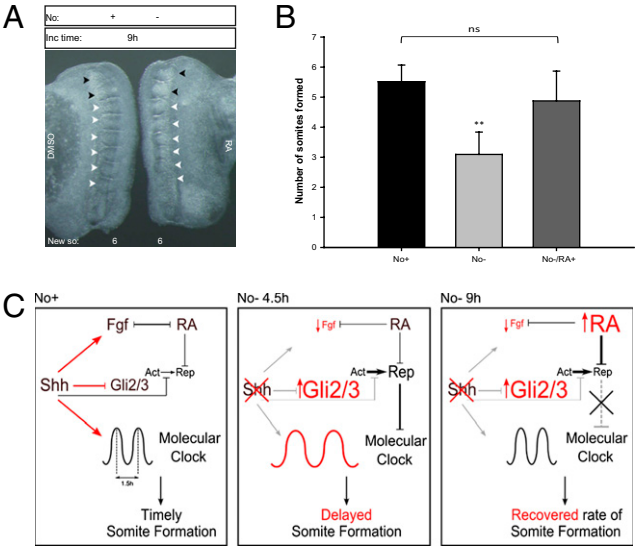


**Fig. 7.** Shh and RA signaling pathways interplay in the PSM to ensure timely somite formation. (*A* and *B*) Retinoic acid mediates the recovery of timely somite formation in absence of notochord. (*A*) Explants with ($No^+$) or without ($No^-$) notochord were cultured for 9 h with DMSO (control explant) or with 15 μM of retinoic acid (RA) (experimental explant). Black arrowheads indicate somites formed before culture; white arrowheads indicate somites formed during incubation period (New so). (*B*) Graphic representation of mean number of somites formed by explants with notochord ($No^+$), without notochord ($No^-$) or without notochord but with RA ($No^-/RA^+$). $^{**}P < 0.05$ vs. $No^+$ or $No^-/RA$. ns, Not statistically significant. Data are mean ± SD. (*C*) Proposed model for molecular events underlying temporal control of somite formation in the presence ($No^+$) or absence ($No^-$) of notochord over time. In the presence of notochord-derived Shh, *gli2/3* expression in the PSM is repressed, and timely molecular clock oscillations and somite formation occur. When Shh signaling is disrupted, *gli2/3* are overexpressed, and there is a delay in both the clock period and PSM morphological segmentation. The normal rate of these events is reestablished over time due to an up-regulation of RA that counteracts Gli activity (detailed in text).

inhibit *gli2* expression (23), this does not occur within our experimental time frame. Because RA also modulates Gli activity (24), our results suggest that *raldh2* overexpression after 9 h of notochord ablation rescues somite formation through an inhibition of Gli2/3 activity in the PSM (Fig. 7*C, Right*). It is plausible that somite formation recovery may further involve the inhibition of *gli2* expression levels later in time. Our work strongly suggests that Shh and RA pathways interplay in the PSM to ensure timely somite formation and positions Gli2/3 at the intersection of these two pathways. Gli2 was recently reported to exert direct transcriptional regulation on the murine *hes* gene in retinal explants (25). In our experimental setup, *gli2* overexpression was concomitant with a deregulation in the *tempo* of *hairy2* transcriptional bursts, suggesting that in the PSM the same transcriptional regulation could take place. Hence, Shh-mediated restriction of Gli activity in the PSM seems to be essential to ensure the pace of both molecular clock oscillations and somite formation. Interestingly, Shh has been recently described to be essential for proper timing of neural progenitor differentiation, and RA was proposed to compensate for Shh deficiency in this developmental system (26).

Somitogenesis is a robust developmental mechanism. This can be achieved only by the integration of multiple signaling pathways. In fact, Notch, Wnt, Fgf, and RA signaling pathways operate in the PSM molecular clock and/or determination front (3, 4, 6). In the present work, we provide conclusive evidence that Shh signaling is a component of the intricate molecular machinery responsible for temporal control of somite formation.

Multiple congenital vertebral malformations have been associated with mutations in somitogenesis clock genes (1, 3). Furthermore, there are reports associating the Shh signaling pathway with congenital vertebral anomalies (27, 28). We report a previously uncharacterized association between these two molecular systems operating in the formation of early embryonic vertebrae precursor structures. A deeper understanding of the multiple molecular interactions occurring during the somitogenesis process is crucial for an adequate scientific contribution to the medical community, striving to better comprehend and treat such human malformations.

## Materials and Methods

Detailed materials and methods can be found in *SI Materials and Methods*. Chick embryos were staged according to Hamburger and Hamilton (HH) (29), and somites were considered to be formed when a definite cleft was observed (30). Antisense digoxigenin-labeled RNA probes were produced as described: *shh* (31), *hairy2* (17), *lunatic fringe* (32), *fgf8* (33), *raldh2* (34), *patched-1* (20), *gli1* and *gli2* (35), and *gli3* (19). A *smoothened*-specific probe was generated. For explant cultures, ventrally positioned embryos were divided into two halves by cutting across the three germ layers at the midline level, either parallel to the embryonic axis or bisecting the axial structures. In Shh-graft experiments, clumps of either QT6-ctrl or QT6-Shh cells (36) were juxtaposed to the $No^{-}$ experimental explants. For chemical treatments, 15 μM of RA (Sigma), 10 μg/mL cyclopamine (Calbiochem), 25 μg/mL forskolin (Sigma), or DMSO was added to the culture medium. In situ hybridization was performed as previously described (37).

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1. Andrade RP, Palmeirim I, Bajanca F (2007) Molecular clocks underlying vertebrate embryo segmentation: A 10-year-old hairy-go-round. *Birth Defects Res C Embryo Today* 81:65–83.
2. Palmeirim I, Henrique D, Ish-Horowicz D, Pourquié O (1997) Avian hairy gene expression identifies a molecular clock linked to vertebrate segmentation and somitogenesis. *Cell* 91:639–648.
3. Dequéant ML, et al. (2006) A complex oscillating network of signaling genes underlies the mouse segmentation clock. *Science* 314:1595–1598.
4. Aulehla A, et al. (2003) Wnt3a plays a major role in the segmentation clock controlling somitogenesis. *Dev Cell* 4:395–406.
5. Diez del Corral R, et al. (2003) Opposing FGF and retinoid pathways control ventral neural pattern, neuronal differentiation, and segmentation during body axis extension. *Neuron* 40:65–79.
6. Dubrulle J, McGrew MJ, Pourquié O (2001) FGF signaling controls somite boundary position and regulates segmentation clock control of spatiotemporal Hox gene activation. *Cell* 106:219–232.
7. Packard DS, Jr (1980) Somitogenesis in cultured embryos of the Japanese quail, Coturnix coturnix japonica. *Am J Anat* 158:83–91.
8. Packard DS, Jr, Jacobson AG (1976) The influence of axial structures on chick somite formation. *Dev Biol* 53:36–48.
9. Packard DS, Jr, Zheng R-Z, Turner DC (1993) Somite pattern regulation in the avian segmental plate mesoderm. *Development* 117:779–791.
10. Ingham PW, McMahon AP (2001) Hedgehog signaling in animal development: Paradigms and principles. *Genes Dev* 15:3059–3087.
11. Fan C-M, Tessier-Lavigne M (1994) Patterning of mammalian somites by surface ectoderm and notochord: Evidence for sclerotome induction by a hedgehog homolog. *Cell* 79:1175–1186.
12. Rong PM, Teillet M-A, Ziller C, Le Douarin NM (1992) The neural tube/notochord complex is necessary for vertebral but not limb and body wall striated muscle differentiation. *Development* 115:657–672.
13. Teillet MA, LaPointe F, Le Douarin NM (1998) The relationships between notochord and floor plate in vertebrate development revisited. *Proc Natl Acad Sci USA* 95: 11733–11738.
14. Chiang C, et al. (1996) Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature* 383:407–413.
15. Varjosalo M, Taipale J (2008) Hedgehog: Functions and mechanisms. *Genes Dev* 22: 2454–2472.
16. Palmeirim I, Rodrigues S, Dale JK, Maroto M (2008) Development on time. *Adv Exp Med Biol* 641:62–71.
17. Jouve C, et al. (2000) Notch signalling is required for cyclic expression of the hairy-like gene HES1 in the presomitic mesoderm. *Development* 127:1421–1429.
18. McGrew MJ, Dale JK, Fraboulet S, Pourquié O (1998) The lunatic fringe gene is a target of the molecular clock linked to somite segmentation in avian embryos. *Curr Biol* 8:979–982.
19. Borycki A-G, Brown AMC, Emerson CP, Jr (2000) Shh and Wnt signaling pathways converge to control Gli gene activation in avian somites. *Development* 127:2075–2087.
20. Marigo V, Tabin CJ (1996) Regulation of patched by sonic hedgehog in the developing neural tube. *Proc Natl Acad Sci USA* 93:9346–9351.
21. Freitas C, Rodrigues S, Charrier JB, Teillet MA, Palmeirim I (2001) Evidence for medial/lateral specification and positional information within the presomitic mesoderm. *Development* 128:5139–5147.
22. Gibb S, et al. (2009) Interfering with Wnt signalling alters the periodicity of the segmentation clock. *Dev Biol* 330:21–31.
23. Ribes V, Le Roux I, Rhinn M, Schuhbaur B, Dollé P (2009) Early mouse caudal development relies on crosstalk between retinoic acid, Shh and Fgf signalling pathways. *Development* 136:665–676.
24. Goyette P, et al. (2000) Regulation of gli activity by all-trans retinoic acid in mouse keratinocytes. *Cancer Res* 60:5386–5389.
25. Wall DS, et al. (2009) Progenitor cell proliferation in the retina is dependent on Notch-independent Sonic hedgehog/Hes1 activity. *J Cell Biol* 184:101–112.
26. Oh S, Huang X, Liu J, Litingtung Y, Chiang C (2009) Shh and Gli3 activities are required for timely generation of motor neuron progenitors. *Dev Biol* 331:261–269.
27. Celli J, van Bokhoven H, Brunner HG (2003) Feingold syndrome: Clinical review and genetic mapping. *Am J Med Genet A* 122A:294–300.
28. Kim PC, Mo R, Hui Cc C (2001) Murine models of VACTERL syndrome: Role of sonic hedgehog signaling pathway. *J Pediatr Surg* 36:381–384.
29. Hamburger V, Hamilton HL (1992) A series of normal stages in the development of the chick embryo. 1951. *Dev Dyn* 195:231–272.
30. Pourquié O, Tam PPL (2001) A nomenclature for prospective somites and phases of cyclic gene expression in the presomitic mesoderm. *Dev Cell* 1:619–620.
31. Riddle RD, Johnson RL, Laufer E, Tabin C (1993) Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell* 75:1401–1416.
32. Sakamoto K, et al. (1997) Identification of a chick homologue of Fringe and C-Fringe 1: Involvement in the neurogenesis and the somitogenesis. *Biochem Biophys Res Commun* 234:754–759.
33. Crossley PH, Minowada G, MacArthur CA, Martin GR (1996) Roles for FGF8 in the induction, initiation, and maintenance of chick limb development. *Cell* 84:127–136.
34. Tsukui T, et al. (1999) Multiple left-right asymmetry defects in Shh(-/-) mutant mice unveil a convergence of the shh and retinoic acid pathways in the control of Lefty-1. *Proc Natl Acad Sci USA* 96:11376–11381.
35. Marigo V, Scott MP, Johnson RL, Goodrich LV, Tabin CJ (1996) Conservation in hedgehog signaling: Induction of a chicken patched homolog by Sonic hedgehog in the developing limb. *Development* 122:1225–1233.
36. Duprez D, Fournier-Thibault C, Le Douarin N (1998) Sonic Hedgehog induces proliferation of committed skeletal muscle cells in the chick limb. *Development* 125: 495–505.
37. Henrique D, et al. (1995) Expression of a Delta homologue in prospective neurons in the chick. *Nature* 375:787–790.

# Supporting Information

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### SI Materials and Methods

**Eggs and Embryos.** Fertilized *Gallus gallus* eggs were incubated at 37 °C in a 49% humidified atmosphere and staged according to Hamburger and Hamilton (HH) (1). A somite was considered to be completely formed when a definite cleft separating it from the PSM was observed (2).

**RNA Probes.** Antisense digoxigenin-labeled RNA probes were produced as previously described: *shh* (3), *hairy2* (4), *lunatic fringe* (5), *fgf8* (6), *raldh2* (7), *patched-1* (8), *patched-2* (9), *gli1* and *gli2* (10), *gli3* (11), *axin2* (12), *pax3* (13), *sprouty1* (14), and *meis2* (15). S*moothened* probe was generated by amplifying a 1,200-bp fragment of chick *smoothened* (XM_414970) using the sense 5′-TACTCACCGTGGCCATCCTG-3′ and antisense 5′-TCCTTC-TTCTTCTTCCTCCGCT-3′ oligos. The construct was confirmed by sequencing.

**Explant Cultures.** Chick embryos in stages HH10–14 were used. Ventrally positioned embryos were divided into two halves by cutting across the three germ layers at the midline level, either parallel to the embryonic axis or bisecting the axial structures (see below). For all explants, a second cut was made above the second formed somite and a third one immediately above the Hensen's node (HN) to prevent the addition of new cells to the PSM over time. Tissues thus delimited and excised created control and experimental explants, which were cultured individually, in a dorsal position, as previously described (16). Similar results were obtained in all experimental conditions tested whether the control/experimental explants were the left or right one. Number of somites formed was assessed after clearing the explants in formamide.

***Notochord ablation.*** A cut was made parallel to the embryonic axis and along the neural tube so that only one explant possessed the notochord and the floor plate of the neural tube. For graft experiments, clumps of either QT6 quail fibroblasts stably transfected with an empty vector (QT6-ctrl) or with a construct carrying the SHH-coding region (QT6-Shh) (17) were juxtaposed to the $No^-$ experimental explants.

In the retinoic acid (RA) treatment experiments, explants without notochord were cultured for 9 h in medium containing 15 μM of RA (Sigma), whereas the controls were incubated with DMSO (18). For FGF pathway induction studies, experimental explants ($No^-$) were incubated in medium containing mouse FGF8 (R&D Systems) at 250 ng/mL (19), whereas controls were incubated in $PBS^-$-containing medium. To inhibit the FGF pathway, explants with notochord ($No^+$) were incubated in medium with 50 μM of SU5402 (Calbiochem), whereas controls were incubated in the presence of DMSO.

***Notochord splitting.*** A longitudinal cut was performed bisecting both neural tube and notochord, producing two equivalent $No^+$ explants. For Shh chemical inhibition, experimental explants were cultured for 9 h in medium with 10 μg/mL cyclopamine (Calbiochem) or 25 μg/mL forskolin (Sigma), whereas the controls were incubated in DMSO-supplemented medium.

In the RA overexpression experiments, explants without notochord were cultured for 9 h in medium containing increasing RA (Sigma) concentrations (15, 30, and 100 μM), whereas the controls were incubated in DMSO-supplemented medium.

For the Shh overexpression studies, clumps of either QT6-ctrl or QT6-Shh cells (17) were juxtaposed to the $No^+$ experimental explants. In the combined SHH/RA treatments, clumps of QT6-Shh cells were juxtaposed to $No^+$ explants and were incubated in medium supplemented with 15 μM of RA.

**In Ovo Microsurgery.** Microsurgery was performed in ovo in embryos staged HH8–9. Indian ink was injected under the embryo to improve contrast, and a longitudinal surgical slit lateral to the notochord was performed, from the last formed somite to HN. Operated embryos were reincubated, after which they were treated for in situ hybridization.

**In Situ Hybridization.** In situ hybridization was performed as previously described (20). Background staining was reduced by using 1× SSC hybridization solution. Explant pairs were processed simultaneously, stained for the same amount of time and photographed using an Olympus SZX16 stereomicroscope.

**In Situ Hybridization on Paraffin Sections.** Stage HH13 or HH9 embryos were collected and fixed overnight in a solution containing 60% ethanol, 30% formaldehyde, and 10% acetic acid. Embryos were dehydrated in a series of ethanol dilutions of ethanol and washed in xylene. Finally, they were embedded in paraffin in the desired orientation. Transversal sections of 10 μm were made using Microm HM325 on SuperFrost Plus (Menzel-Glaser) slides and allowed to dry at 37 °C overnight. In situ hybridization on paraffin sections was performed as described previously (21).

**Cross-Section Preparation.** Hybridized embryos were embedded in Technovit 7100 (Heraus Kulzer GmbH) and sectioned using the automatic microtome Leica RM2155. Mounted sections were photographed using Olympus B BX61 microscope.

**TUNEL Assay.** Apoptosis was analyzed using the Cell Death Detection Kit (Roche) in explants with or without notochord, incubated for 9 or 12 h. Explants were fixed overnight in 4% paraformaldehyde (PFA) in PBS, permeabilized with PBS/0.5% Triton X-100/0.1% sodium citrate for 20 min at room temperature, and washed in PBS. Positive control embryos were incubated with DNase at 37 °C for 1 h. Embryos and explants were incubated overnight at 37 °C with the TUNEL solution mix and washed at least three times in PBS before visualization.

**PH3 Immunohistochemistry (IHC).** Whole-mount IHC for PH3 was performed in explants with or without notochord, incubated for 9 or 12 h to analyze cell proliferation. Explants were fixed in 4% PFA in PBS and permeabilized with 0.5% Triton X-100 in PBS for 3 h. Explants were incubated in blocking solution (PBS 1% Triton/10% FBS/0.2% sodium azide) twice for 1 h. Endogenous peroxidases were block by incubating explants in blocking solution containing 0.1% H2O2. Primary antibody incubation with rabbit anti-phospho-histone H3 (UBI) (1/100, diluted in blocking solution) was performed overnight at 4 °C, followed by washes in blocking solution. Explants were washed in PBS 1% Triton to remove all azide, followed by secundary antibody incubation overnight at 4 °C (biotinylated goat anti-polyvalent, Ultravision detection system; Lab Vision). After PBS 1% Triton washes, explants were incubated in DAB substrate (Ultravision detection system; Lab Vision) for 2 h. Fresh DAB plus $H_2O_2$ was then added. Explants were incubated until staining reached the desired intensity and were washed with PBS.

**Time-Lapse Imaging.** For time-lapse experiments, embryos at stages HH3–5 were removed from the egg, placed in New culture (22), and reincubated until six to eight somites stage (HH8–9). In each embryo, a small incision was made lateral to the notochord across the three germ layers and along the length of four to seven pre-

sumptive somites. Embryo cultures were maintained at 37.5 °C and 90% humidity and observed using a Leica DMIRE2 inverted microscope equipped with a Hamamatsu EM-CCD digital camera. Images were acquired with a 2.5× NA 0.07 objective using Image-Pro Plus software (Media Cybernetics). Photographs were collected every 5 min during a period of 10 h of incubation. Image series were recorded as TIFF files and processed using ImageJ software (http://rsb.info.nih.gov/ij/) to correct embryo drift and to adjust contrast and brightness.

**Statistical Analysis.** Quantitative data are presented as mean value ± SD and were analyzed using the SigmaStat3.5 software (Systat Software, Inc.). Statistical significance was set at $P < 0.01$ or $P < 0.05$, as indicated.


1. Hamburger V, Hamilton HL (1992) A series of normal stages in the development of the chick embryo. 1951. *Dev Dyn* 195:231–272.
2. Pourquie O, Tam PPL (2001) A nomenclature for prospective somites and phases of cyclic gene expression in the presomitic mesoderm. *Dev Cell* 1:619–620.
3. Riddle RD, Johnson RL, Laufer E, Tabin C (1993) Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell* 75:1401–1416.
4. Jouve C, et al. (2000) Notch signalling is required for cyclic expression of the hairy-like gene HES1 in the presomitic mesoderm. *Development* 127:1421–1429.
5. Sakamoto K, et al. (1997) Identification of a chick homologue of Fringe and C-Fringe 1: Involvement in the neurogenesis and the somitogenesis. *Biochem Biophys Res Commun* 234:754–759.
6. Crossley PH, Minowada G, MacArthur CA, Martin GR (1996) Roles for FGF8 in the induction, initiation, and maintenance of chick limb development. *Cell* 84:127–136.
7. Tsukui T, et al. (1999) Multiple left-right asymmetry defects in Shh2/2 mutant mice unveil a convergence of the Shh and retinoic acid pathways in the control of Lefty-1. *Proc Natl Acad Sci USA* 96:11376–11381.
8. Marigo V, Tabin CJ (1996) Regulation of Patched by Sonic hedgehog in the developing neural tube. *Proc Natl Acad Sci USA* 93:9346–9351.
9. Pearse RV, Vogan KJ, Tabin CJ (2001) Ptc1 and Ptc2 transcripts provide distinct readouts of hedgehog signaling activity during chick embryogenesis. *Dev Biol* 239:15–29.
10. Marigo V, Scott MP, Johnson RL, Goodrich LV, Tabin CJ (1996) Conservation in hedgehog signaling: Induction of a chicken patched homolog by Sonic hedgehog in the developing limb. *Development* 122:1225–1233.
11. Borycki A-G, Brown AMC, Emerson JCP (2000) Shh and Wnt signaling pathways converge to control Gli gene activation in avian somite. *Development* 127:2075–2087.
12. Gibb S, et al. (2009) Interfering with Wnt signalling alters the periodicity of the segmentation clock. *Dev Biol* 330:21–31.
13. Goulding MD, Lumsden A, Gruss P (1993) Signals from the notochord and floor plate regulate the region-specific expression of two Pax genes in the developing spinal cord. *Development* 117:1001–1016.
14. Eloy-Trinquet S, Wang H, Edom-Vovard F, Duprez D (2009) Fgf signaling components are associated with muscles and tendons during limb development. *Dev Dyn* 238: 1195–1206.
15. Heine P, Dohle E, Bumsted-O'Brien K, Engelkamp D, Schulte D (2008) Evidence for an evolutionary conserved role of homothorax/Meis1/2 during vertebrate retina development. *Development* 135:805–811.
16. Palmeirim I, Henrique D, Ish-Horowicz D, Pourquie O (1997) Avian hairy gene expression identifies a molecular clock linked to vertebrate segmentation and somitogenesis. *Cell* 91:639–648.
17. Duprez D, Fournier-Thibault C, Le Douarin N (1998) Sonic Hedgehog induces proliferation of committed skeletal muscle cells in the chick limb. *Development* 125: 495–505.
18. Diez del Corral R, et al. (2003) Opposing FGF and retinoid pathways control ventral neural pattern, neuronal differentiation, and segmentation during body axis extension. *Neuron* 40:65–79.
19. Olivera-Martinez I, Storey KG (2007) Wnt signals provide a timing mechanism for the FGF-retinoid differentiation switch during vertebrate body axis extension. *Development* 134:2125–2135.
20. Henrique D, et al. (1995) Expression of a Delta homologue in prospective neurons in the chick. *Nature* 375:787–790.
21. Tozer S, et al. (2007) Involvement of vessels and PDGFB in muscle splitting during chick limb development. *Development* 134:2579–2591.
22. New DAT (1955) A new technique for the cultivation of the chick embryo in vitro. *J. Embryol Exp Morph* 3:326.

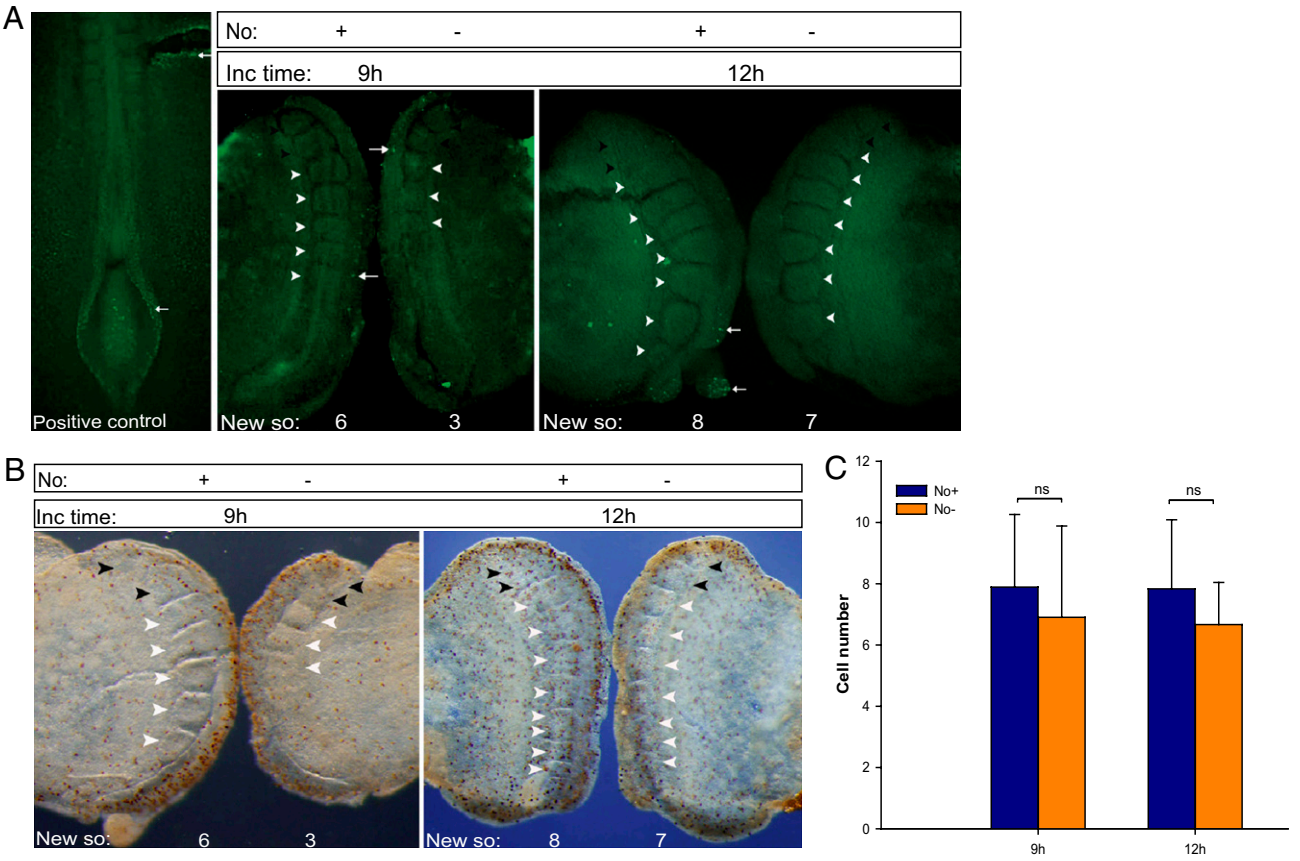



**Fig. S1.** Alterations in levels of cell death or proliferation cannot account for somite formation delay in notochord-ablated PSMs. (*A*) PSM cell apoptosis was assessed by performing TUNEL assay in explants with ($No^+$) or without ($No^-$) notochord incubated separately for 9 h or 12 h. A positive control for the assay is shown. White arrows point to specific TUNEL staining. (*B*) Explants with ($No^+$) or without ($No^-$) notochord and incubated for 9 h or 12 h were processed for immunohistochemistry with PH3 antibody. Number of somites formed during culture (New so) is indicated. Black arrowheads indicate somites formed before culture; white arrowheads point to somites formed during the incubation period. (*C*) Graphic representation of PH3 staining in explants with ($No^+$) or without ($No^-$) notochord ($n = 3$). Data are mean ± SD. ns, not statistically significant.

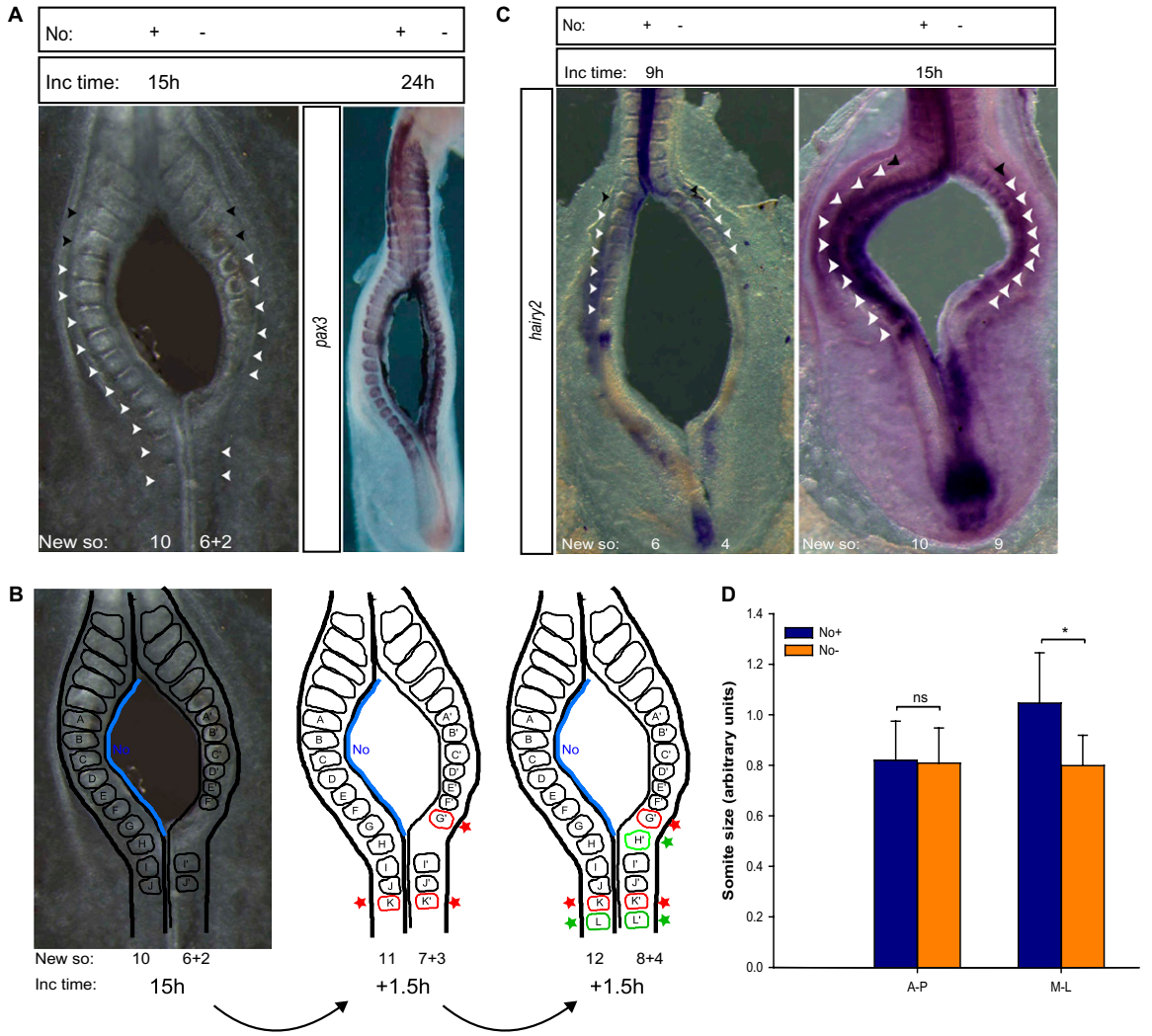



**Fig. S2.** Notochord ablation in ovo delays, but does not halt, somite segmentation of undetermined PSM. (*A*) Representative results obtained in in ovo operated embryos after a reincubation period of 15 h and 24 h, showing incomplete and complete segmentation of notochord-ablated tissue, respectively. Embryos incubated for 24 h were processed for *pax3* in situ hybridization for somite visualization. (*B*) Representation of how somite number is recovered in $No^-$ PSM tissue. Figure of 15-h incubated embryo has been outlined, and somites formed upon incubation are marked alphabetically. During the incubation period, timely somite formation proceeded in the $No^+$ PSM, reaching the tissue caudal to the slit, where timely bilateral somite formation was resumed. Meanwhile, there is a delay in somite segmentation in the notochord-deprived PSM, resulting in a gap of unsegmented tissue of approximately a 2-somite length at the posterior end of the slit. When somite formation rate is recovered in $No^-$ PSM, timely somite formation proceeds in the unsegmented gap, so that during the next 1.5 h a new pair of somites will be formed in the PSM (somites K-K′, red), concomitantly with an extra somite in the No- tissue facing the surgical slit (somite G′, red). The same is true for the next 1.5 h: Somite pair L-L′ is formed, as well as somite H′ (green). (*C*) In ovo operated embryos in situ hybridized for *hairy2* after reincubation periods of 9 h and 15 h, showing concomitant deregulation of molecular clock with somite formation delay. Presence ($No^+$) or absence ($No^-$) of notochord, and number of somites formed during reincubation (New so), are indicated. Black arrowheads indicate somites formed before culture; white arrowheads indicate somites formed during the incubation period. (*D*) Graphic representation of mean size of somites formed in the presence ($No^+$) or absence ($No^-$) of the notochord, along both anterior–posterior (A-P) and medial–lateral (M-L) axes. Data are mean ± SD. $*P < 0.01$. ns, not statistically significant.

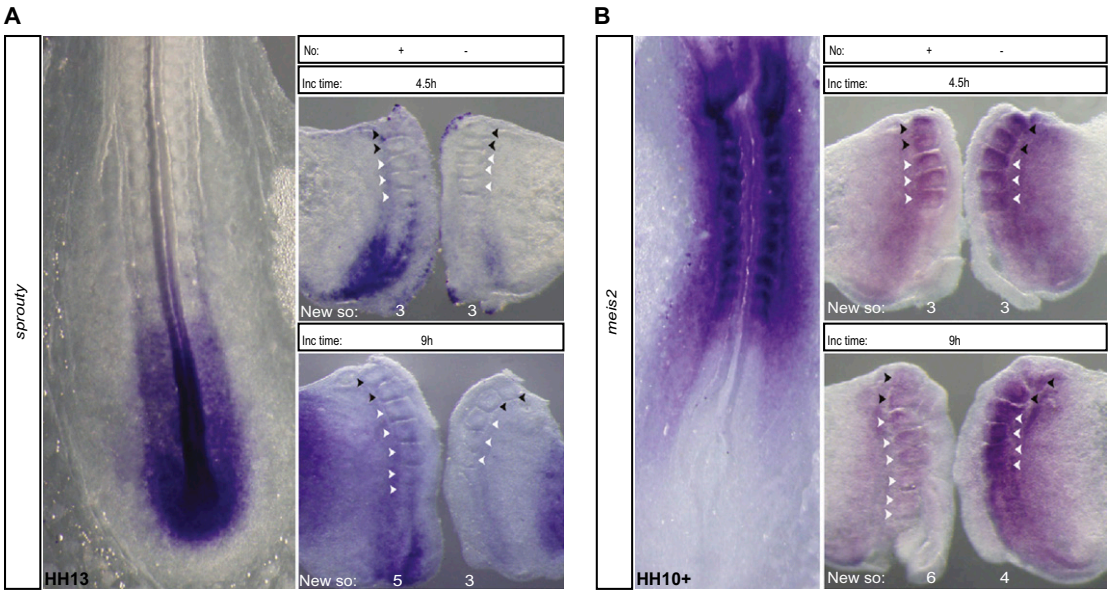



**Fig. S3.** Notochord ablation alters FGF and RA signaling readouts. (*A* and *B*) Expression patterns of *sprouty* and *meis2*, downstream targets of FGF and RA, respectively, evaluated by in situ hybridization in explants cultured for 4.5 or 9 h. Presence ($No^+$) or absence ($No^-$) of notochord is indicated. Black arrowheads indicate somites formed before culture; white arrowheads indicate somites formed during incubation (New so).

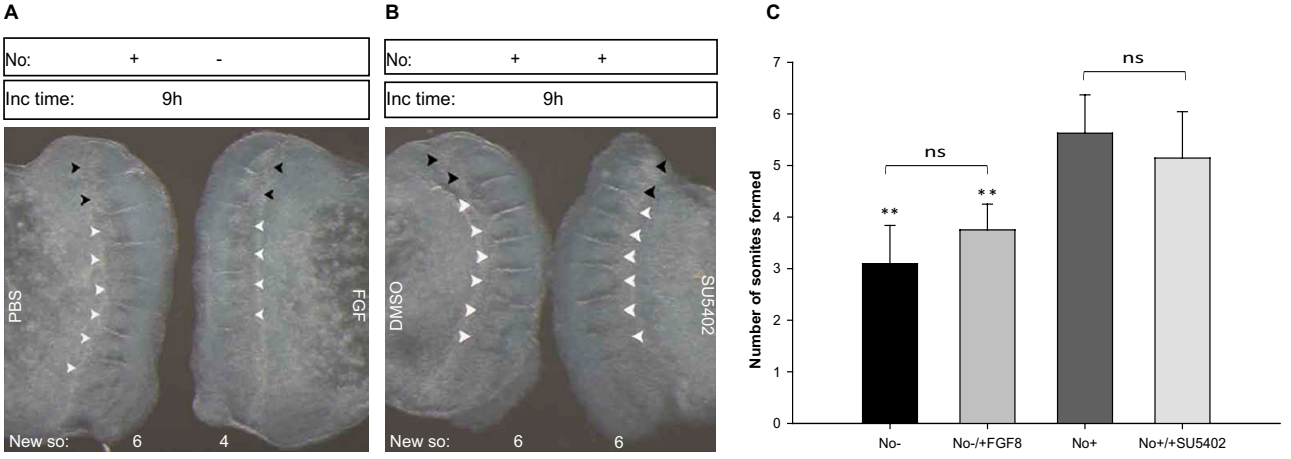



**Fig. S4.** Somite formation delay in absence of notochord is primarily due to an impairment of FGF signaling. (*A*) Explants with ($No^+$) or without ($No^-$) notochord were cultured for 9 h with PBS (control explant) or with 250 ng/mL FGF8 (experimental explant). (*B*) Explants with ($No^+$) notochord were cultured for 9 h with DMSO (control explant) or with 50 μM SU5401 (experimental explant). Black arrowheads indicate somites formed before culture; white arrowheads indicate somites formed during incubation period (New so). (*C*) Graphic representation of mean values of the number of somites formed by explants without notochord ($No^-$), without notochord incubated with FGF8 ($No^-$/+FGF), with notochord ($No^+$), or with notochord and SU5402 ($No^+$/+SU5402). $^{**}P < 0.05$ vs. $No^+$ or $No^+$/+SU5402. ns, not statistically significant. Data are mean ± SD.

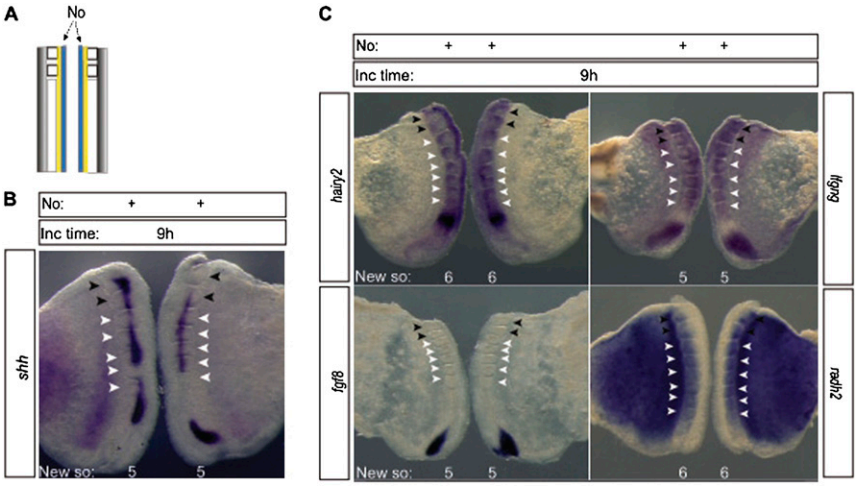



**Fig. S5.** Presence of notochord ensures timely somite formation and molecular segmentation. (*A*) Symmetrical explants, both containing notochord tissue, were generated by surgically cutting both the neural tube and notochord down the midline. (*B*) Because of the harshness of the notochord, it was not always possible to perfectly bisect it, as denoted by hybridization with notochord marker *shh*. (*C*) Although sometimes discontinuous, the presence of notochord tissue ensures normal somite segmentation rate, as 98% of the explants pairs present the same number of somites after a 9-h incubation ($n = 34$), in contrast to only 5% when the notochord is confined to half ($n = 90$). Whole-mount in situ hybridization of these explants clearly shows that the molecular clock genes *hairy2* and *lfng* present similar expression patterns in symmetrical explants, and no alterations are observed for *fgf8* and *raldh2*. Presence of notochord ($No^+$) and number of somites formed (New so) are indicated. Black arrowheads indicate somites formed before culture; white arrowheads point to somites formed during the incubation period.

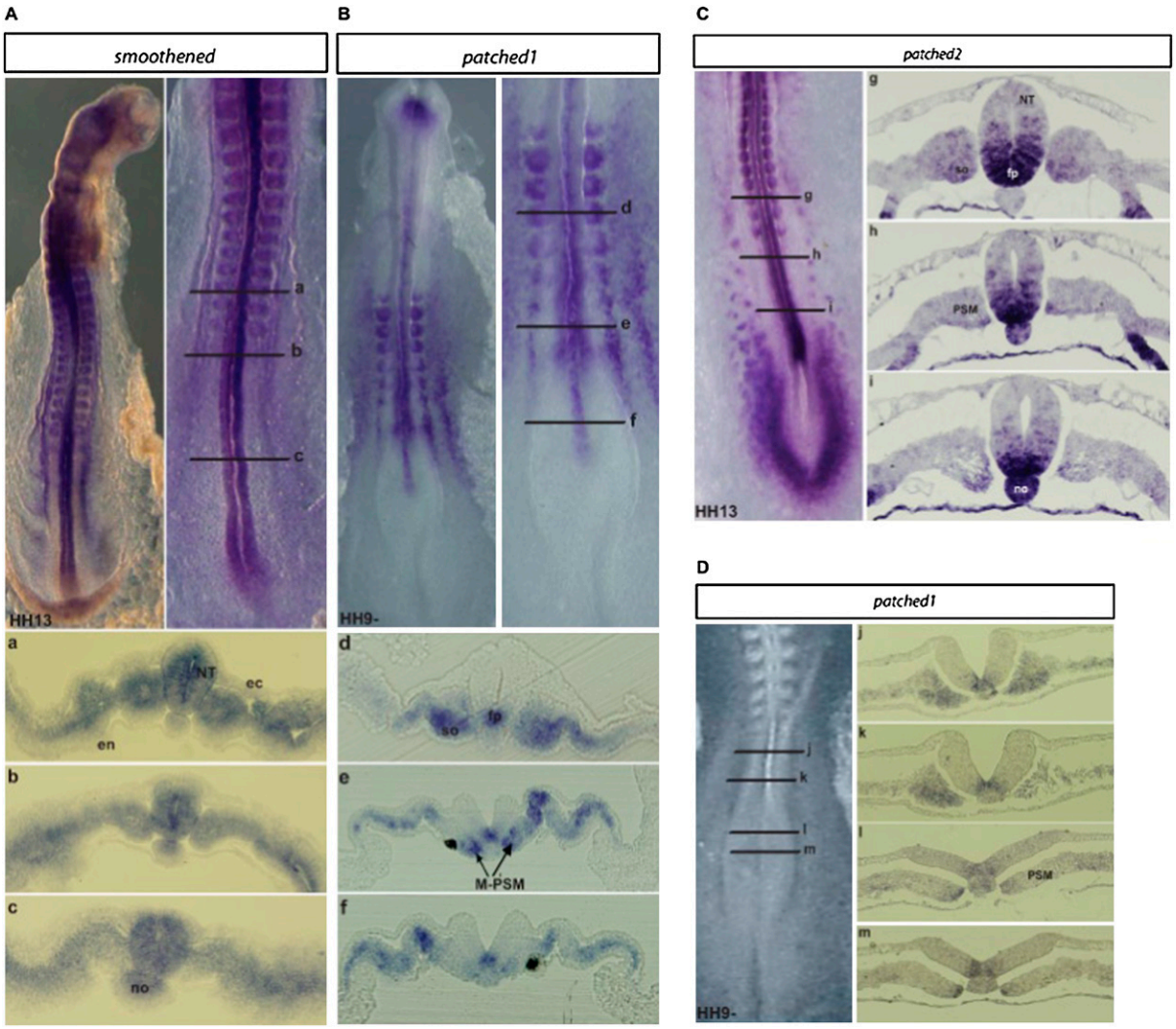



**Fig. S6.** Expression patterns of Shh signaling components *smoothened*, *patched1,* and *patched2*. Chicken embryos from HH9-15 were processed for in situ hybridization using a probe for *smoothened, patched1,* or *patched2,* and representative results are shown. (*A*) *Smo* is expressed in neural tube (NT) and throughout all mesoderm-derived tissues, including PSM and notochord (no), but is absent in ectoderm (ec) and endoderm (en). In epithelialized tissues, *smo* is preferably localized in the cells' apical region (Aa). (*B*) *Ptch1* is expressed in the ventral part of somites (so) and neural tube floor plate (fp). Upon increased staining, *ptch1* was also visible in the most medial PSM (M-PSM), just adjacent to notochord (Be). (*C*) In situ hybridization in paraffin sections shows that *ptch2* is expressed in the ventral part of somites (so), neural tube floor plate (fp), notochord (no), and also in the most medial portion of the PSM. (*D*) In situ hybridization in paraffin sections suggests an anterior–posterior gradient of *ptch1* expression in M-PSM region. Black lines indicate anterior–posterior levels of transverse sections.

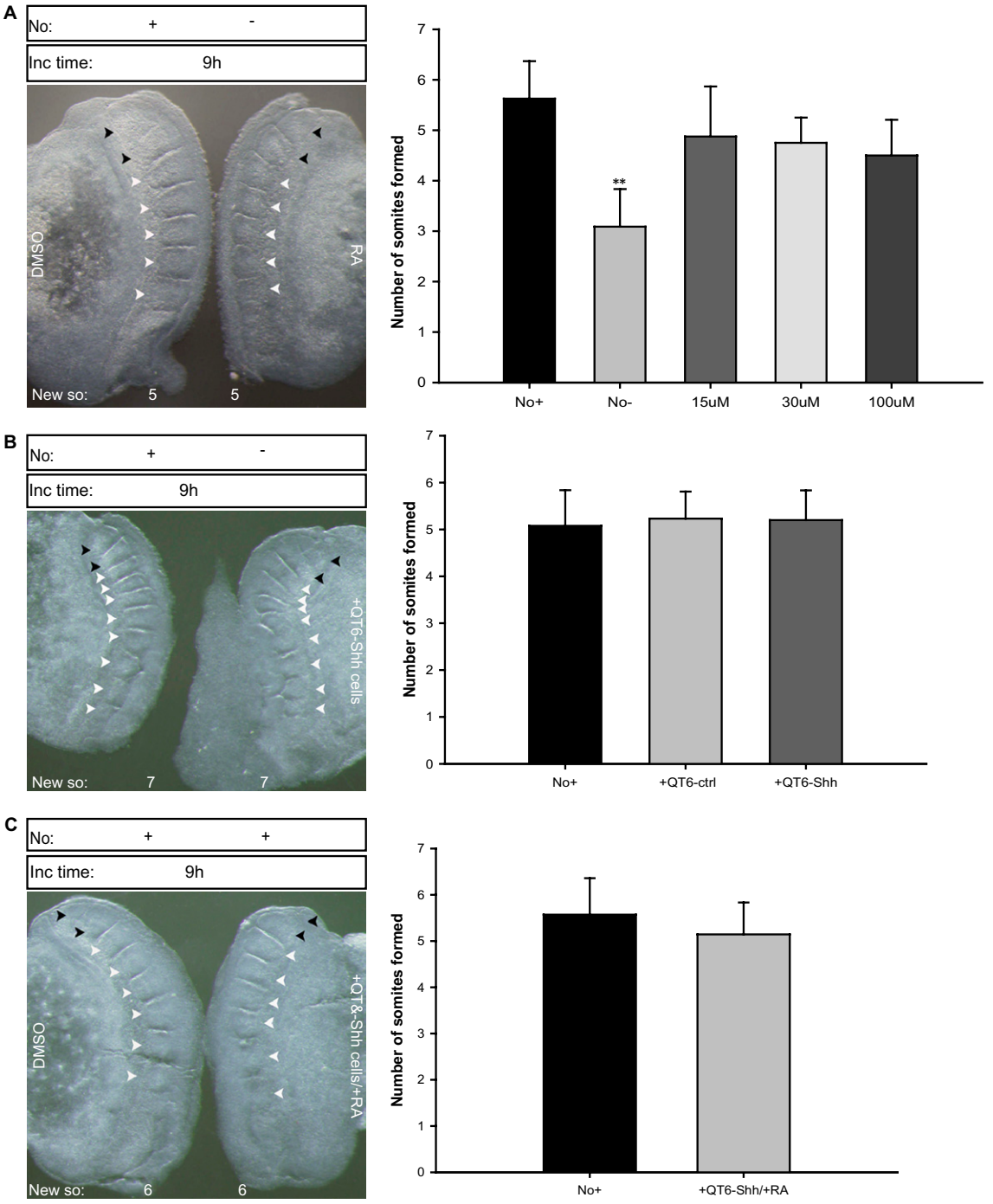



**Fig. S7.** Overexpression of RA, Shh or both does not lead to a faster PSM segmentation rate. (*A*) Explants with ($No^+$) or without ($No^-$) notochord were cultured for 9 h with DMSO (control explant) or with increasing concentrations of retinoic acid (RA) (experimental explant). Graphic representation of mean number of somites formed by explants with notochord ($No^+$), without notochord ($No^-$), or without notochord but with increasing concentrations of RA (μM). $^{**}P < 0.05$ vs. $No^+$ or different RA concentrations. (*B*) Explants with ($No^+$) notochord alone (control explant) or juxtaposed to a clump of QT6-producing Shh cells (QT6-Shh, experimental explant) were cultured for 9 h. Graphic representation of mean number of somites formed by explants with notochord ($No^+$) and with notochord and juxtaposed control (+QT6-ctrl) or Shh-producing (+QT6-Shh) cells. (*C*) Explants with ($No^+$) notochord (control explant) or with a clump of QT6-producing Shh cells and 15 μM of RA (+QT6-Shh/+RA, experimental explant) were cultured for 9 h. Graphical representation of mean values of number of somites formed by explants with notochord ($No^+$) or with notochord plus QT6-Shh cells and RA (+QT6-Shh/+RA). Data are represented as mean ± SD. Black arrowheads indicate somites formed before culture; white arrowheads indicate somites formed during incubation period (New so).


**Fig. S8.** Somite formation delay in absence of Shh is not due to impairment of Wnt signaling pathway. Representative results of *axin2* expression in HH13 whole embryo and in $No^+$/No- explants incubated for 4.5 h and 9 h. Presence ($No^+$) or absence ($No^-$) of notochord and number of somites formed during reincubation (New so) are indicated. Black arrowheads indicate somites formed before culture; white arrowheads point to somites formed during incubation period. Expression of *axin2* is unaltered in 60% of notochord-ablated explants, and expression in the other 40% is only slightly down-regulated after 4.5 h. After 9 h, *axin2* is clearly down-regulated in all explants tested. *Axin2* down-regulation after 9 h of incubation is a predictable result from our proposed model (Fig. 7), according to which Gli2/3 repressor forms are abundant in PSM up to this time due to the absence of Shh signaling; Gli3R has been shown to inhibit *axin2* expression (1), so *axin2* down-regulation seems likely to be a consequence of Shh deprivation. Some reports position Shh pathway upstream of Wnt (2, 3) and our results suggest that this could be the case in the PSM, although we do not wish to argue such at this time. It is also very unlikely that a reestablishment of Wnt signaling is the primary event leading to the recovery of timely PSM segmentation after 9 h of Shh deprivation, as *axin2* is down-regulated at this time point. Overall, the results strongly suggest that Wnt signaling is not the primary cause of the segmentation delay observed in the absence of Shh.

1. Ulloa F, Itasaki N, Briscoe J (2007) Inhibitory Gli3 activity negatively regulates Wnt/beta-catenin signaling. *Curr Biol* 17:545–550.
2. Alvarez-Medina R, Le Dreau G, Ros M, Martí E (2009) Hedgehog activation is required upstream of Wnt signalling to control neural progenitor proliferation. *Development* 136: 3301–3309.
3. Miyagawa S, et al. (2009) Dosage-dependent hedgehog signals integrated with Wnt/beta-catenin signaling regulate external genitalia formation as an appendicular program. *Development* 136:3969–3978.

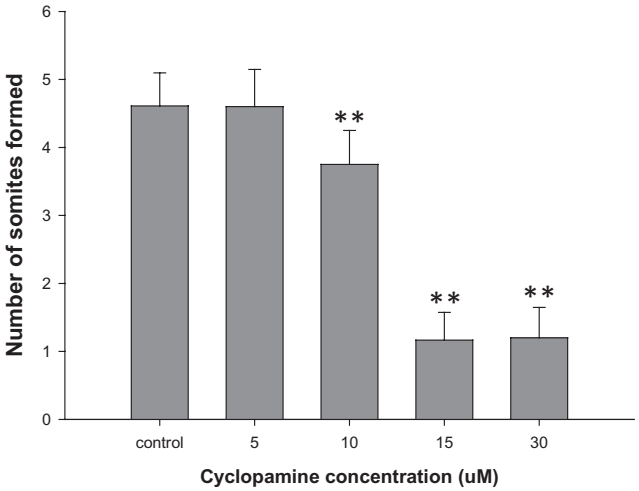
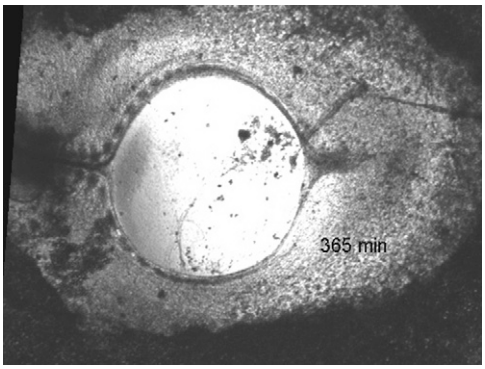



**Fig. S9.** Shh inhibition delays somite formation in a dose-dependent manner. Graphic representation of mean number of somites formed by explants with notochord ($No^+$), in the presence of either DMSO (control) or increasing concentrations of cyclopamine after a 9-h incubation. $^{**}P < 0.05$ vs. control. Data are represented as mean ± SD.




**Movie S1.** Real-time imaging of somite formation delay in notochord-deprived PSM. A small incision was made lateral to the notochord across the three germ layers and along the length of four to seven presumptive somites in six- to eight-somite–stage embryos grown in New culture (1). Embryo cultures were placed in a custom-built air-heated microscope chamber and maintained at 37.5 °C and 90% humidity. Somite formation was imaged over time, and a representative result is shown. In this case, the slit included anteriorly two previously formed somites. The segmentation of the three first somites formed after the microsurgical incision proceeded at the same rate in both PSMs ($No^+$, top; $No^-$, bottom). The formation of the subsequent somites was delayed in the PSM deprived of the notochord/floor plate, whereas the $No^+$ PSM segmented at the normal rate of one somite/90 min.

Movie S1

# CHAPTER3. SOMITE FORMATION DEPENDENCE ON SONIC HEDGEHOG SIGNALING

The results presented in this chapter will constitute another publication:

Tatiana P. Resende, Catarina Freitas, Raquel P. Andrade and Isabel Palmeirim. The role of Shh in PSM mediolateral specification: a two-step process. (*in final phase of preparation).*

**The role of Shh in PSM Medial-Lateral specification: a two-step process**

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Keywords: somitogenesis, PSM, Shh

**Abstract**

In vertebrate embryo development, segmentation relies on the progressive formation of early embryonic vertebrae precursor structures – somites - that periodically bud off from the rostral presomitic mesoderm (PSM). In the chick embryo, a pair of somites is formed every 90min and this periodicity is regulated by cyclic expression of somitogenesis clock genes. We have previously shown that only the medial-most PSM (M-PSM) cells possess intrinsic information for both somite formation and molecular clock gene expression. Recently, we showed that notochord-produced *sonic hedgehog* (*shh*) integrates this regulatory molecular network. In the present work, we evaluated the contribution of axial Shh signaling in the specification of medial and lateral PSM tissue. By performing quail/chick grafts we show that the prospective M- and L- PSM territories are distinctly committed for somite formation. We also show that notochord-derived Shh is the signaling molecule responsible for the recruitment of lateral cells by medial ones in the anterior PSM. Furthermore, our results suggest that the same can be occurring at the prospective PSM territory – different levels of Shh signaling specify distinct PSM fates. Altogether, our results indicate that during somitogenesis Shh is required for both PSM cell fate specification and for correct M-L morphological somite formation from the determined PSM tissue. The results here presented further implicate Shh signaling in cell fate positional information and in the regulatory network underlying somite formation regulation in vertebrate embryo development.

**Introduction**

A characteristic of vertebrate embryo development is the process of somitogenesis, whereby somites, early embryonic structures that will later give rise to vertebrae, intervertebral disks, ribs, the dermis of the back and all the striated muscles of the adult body are progressively formed along the anterior-posterior (A-P) body axis (reviewed in Andrade et al., 2007). Somitogenesis is a fine-tuned regulated mechanism that occurs with a strict coordination, during which somites periodically bud off bilaterally from the rostral portion of the presomitic mesoderm (PSM) flanking the axial organs, notochord and neural tube. Somites are formed in a timely controlled manner and in the trunk region of the chick embryo a new pair of somites is formed every 90min. The periodicity of somite formation is controlled by an embryonic molecular clock, first evidenced by the cyclic expression of *hairy1* gene in chick PSM cells with the same periodicity as somite formation (Palmeirim et al., 1997). Similar oscillatory behavior has been observed in many other genes belonging to signaling pathways such as Notch, Wnt and Fgf (Dequeant et al., 2006;

reviewed in Andrade et al., 2007). Although the molecular clock is operating along the entire PSM, only the anterior third of this tissue is already determined to form somites (Dubrulle et al., 2001). In fact, a wavefront of PSM maturation is defined by opposing molecular gradients with cross-regulatory activities: high levels of FGF/Wnt maintain the posterior PSM in an undetermined state and are counteracted by an opposing gradient of Retinoic Acid (RA) (Dubrulle et al., 2001; Aulehla et al., 2003; Diez del Corral et al., 2003). The intersection of these gradients occurs at the level of somites –III to –IV (Christ and Ordahl, 1995; Dubrulle et al., 2001), defining the so-called determination front, and is displaced caudally over time concomitant with posterior embryo elongation and anterior morphological somite formation.

Fate map studies have shown that PSM cell precursors (P-PSM) are already organized along the anterior-posterior (A-P) axis of the primitive streak: more anterior regions give rise to more medial PSM (M-PSM) cells while more posterior ones contribute to the lateral-most PSM (L-PSM) (Selleck and Stern, 1991; Schoenwolf et al., 1992; Psychoyos and Stern, 1996; Sawada and Aoyama, 1999; Freitas et al., 2001; Eloy-Trinquet and Nicolas, 2002; Iimura et al., 2007). Therefore, medial and lateral portions of a single somite are produced at distinct times during gastrulation. In fact, PSM can be subdivided into medial and lateral compartments, which have different developmental fates. The medial portion gives rise to the the vertebral column, the epaxial muscles that surround the axial body skeleton and the dermis of the back while the lateral section contributes to the hypaxial musculature of the ribs, abdomen and limbs (Olivera-Martinez et al., 2000). Thus, all segmented structures of the vertebrate body arise from the medial PSM portion. Accordingly, previous work from our group indicates that M- and L- PSM possess different intrinsic information for both somite formation and molecular clock gene expression (Freitas et al., 2001). This work showed that M-PSM cells are able to undergo both morphologic and molecular segmentation when isolated from their lateral counterparts while L-PSM does not segment when separated from the medial PSM domain and loses molecular clock gene expression. This is true even for the tissue located above the determination front (Freitas et al., 2001). These results evidence clearly distinct properties of such closely located cells and suggest that M-PSM cells are the ones containing the information for segmentation and are responsible for instructing and recruiting more lateral ones for somite formation.

We have recently shown that in the absence of notochord-derived Sonic hedgehog (Shh) signaling, both somite formation and molecular clock gene oscillations are severely delayed, implicating Shh signaling pathway in the segmentation clock regulatory network (Resende et al., 2010). Sonic hedgehog (Shh) is a morphogen produced by the notochord and neural tube floor plate, controlling

multiple essential vertebrate developmental processes (reviewed in Varjosalo and Taipale, 2008). Shh signaling transduction requires the activity of two membrane-bound receptors, Patched (Ptch) and Smoothened (Smo). Binding of Shh to Ptch releases Smo repression thus activating a transduction cascade that ultimately regulates the Gli family of transcription factors (reviewed in Varjosalo and Taipale, 2008). Shh knock-out mice present an absence of the spinal column and most of the ribs, implicating this molecule in vertebrate skeletal patterning (Chiang et al., 1996). In fact, Shh is required for somite sclerotome induction (Fan and Tessier-Lavignet, 1994), survival of sclerotomal and medial dermomyotomal somitic cells (Rong et al., 1992; Teillet et al., 1998) and, at the PSM level, for timely molecular clock oscillations and somite formation (Resende et al., 2010).

In the present work, we evaluated Shh signaling involvement in the specification of M- and L-PSM tissue fate. By performing *in vitro* explant culture, we show that in the anterior PSM diffusible Shh is the molecule required for the recruitment of lateral cells by medial ones for somite segmentation. Moreover, quail/chick grafts experiments demonstrate that the prospective M- and L- PSM territories are distinctly committed for somite formation and this is probably mediated by differential Shh signaling levels in these domains. Taken together, these results suggest that Shh has a dual function in somitogenesis: elevated Shh levels specifies the prospective M-PSM compartment in the embryo caudal region and secondly, high Shh activity in the anterior PSM enables medial cells to recruit more lateral ones for somite formation. These results further implicate Shh signaling pathway in somitogenesis regulation, constituting an additional level of regulation contributing to the robustness of this developmental event.

## Materials and Methods

### Eggs and embryos

Fertilized *Gallus gallus* eggs were incubated at 37ºC in a 49% humidified atmosphere and staged according to Hamburger and Hamilton (HH) (Hamburger and Hamilton, 1992). A somite was considered to be completely formed when a definite cleft separating it from the PSM was observed (Pourquie and Tam, 2001).

### RNA probes

Antisense digoxigenin-labelled RNA probes were produced as previously described: *bmp4* (Francis et al., 1994), *delta1* (Henrique et al., 1995), *shh* (Riddle et al., 1993), *patched1* (Marigo and Tabin, 1996) and *patched2* (Pearse et al., 2001).

**Whole-mount *in situ* hybridization**

For whole-mount *in situ* hybridization, embryos and explants were fixed overnight at 4ºC in fresh 4% formaldehyde-2mM EGTA in phosphate-buffered saline (PBS), rinsed in PBT (PBS, 0,1% Tween 20), dehydrated through a methanol series and stored in 100% methanol at -20ºC. Hybridization was performed according to the previously described procedure (Henrique et al., 1995). For some probes, background staining was reduced by using hybridization solution containing 1xSSC. All explant pairs were processed simultaneously and stained for the same amount of time. Embryos and explants were photographed in PBT using Olympus SZX16 stereomicroscope.

***In situ* hybridization on Paraffin Sections**

Stage HH9- embryos were collected and fixed overnight in a solution containing 60% ethanol, 30% formaldehyde, and 10% acetic acid. Embryos were dehydrated in a series of ethanol dilutions and washed in xylene. Finally, they were embedded in paraffin in the desired orientation. Transversal sections of 20 μm were made using Microm HM325 on SuperFrost Plus (Menzel-Glaser) slides and allowed to dry at 37 °C overnight. *In situ* hybridization on paraffin sections was performed as described previously (Tozer et al., 2007).

**Generation of quail/chick chimeras**

Quail donor embryos were collected in PBS and put aside while preparing the host. The host chick embryos were incubated for different time periods and operated *in ovo*. The region to be grafted was precisely delimited both in donor and host embryos and subsequently removed from the host and replaced by the donor counterpart with the help of a narrow-tipped Pasteur pipette. After the transplantation, the excess of PBS was gently sucked out with a Pasteur pipette to promote the adhesion of the graft to host tissues. The following day, chimeric embryos were collected, fixed overnight at 4ºC in 4% formaldehyde and processed for immunohistochemistry.

<u>Localization of PL-PSM territory</u>

Chicken and quail eggs were incubated for 30 to 36 hours in order to obtain HH9- stage embryos. Quail midline fragments extending 100-150 μm rostrally from the posterior limit of the open neural plate were grafted homotopically into stage-matched chick hosts. Chimeric embryos were

incubated overnight, after which they were collected and processed for immunohistochemistry in histological sections.

PL- and PM -PSM heterotopic grafts

For the generation of quail/chick chimeras containing two PL-PSMs, quail primitive streak fragments equivalent to the PL-PSM territory were precisely delimited and removed and carefully transferred into a stage-matched chick host. This tissue was grafted into the host's PM-PSM domain, which had been previously excised and removed by a similar procedure. The reverse grafting was also performed: the same procedure was followed but in this case the PM-PSM of the quail donor was removed and grafted into the host's PL-PSM territory which had been previously excised. This generated chimeras in which the prospective PSM territory is composed of two PM-PSM domains. Operated embryos were incubated overnight, after which they were collected, fixed overnight at 4ºC in 4% formaldehyde and processed for immunohistochemistry.

**_In ovo_ Microsurgery**

Fertilized chick eggs were incubated horizontally for *in ovo* microsurgery. The eggshell was cleaned with 70% ethanol, and 0.5 millilitres (ml) of albumen were withdrawn and discarded using a syringe inserted into the blunt end of the eggshell. A window was opened in the eggshell top and a mixture of indian ink:PBS was tangentially injected under the embryo to improve contrast. The vitelline membrane was pulled apart with a tungsten microscalpel to access the embryo. PBS drops were regularly placed on top of the embryo to prevent dehydration. After operation, the eggshell was sealed with plastic tape and embryos were re-incubated at 37°C for distinct periods of time.

*In ovo* barrier insertion

HH12-14 stage chick embryos, incubated in a horizontal position, were used. A slit was performed subdividing longitudinally the PSM into medial and lateral halves. A barrier of suitable size was inserted through the three germ layers of the embryo separating them. Two types of barriers were used: an impermeable tantalum foil barrier or a permeable Millipore filter (0.8 μm) barrier. The embryos were then re-incubated for 3 to 6 hours and subsequently processed for in situ hybridization.

*In ovo* tissue ablation

Selective ablation of PM-PSM or PL-PSM territories was performed in HH9- stage embryos. For the PM-PSM ablation, a primitive streak fragment extending 100-150 μm caudally from the

posterior limit of the median pit was removed; for PL-PSM excision the region removed extended 100-150 µm rostrally from the caudal limit of the open neural plate. Each domain was carefully delimited using a steel microscalpel and removed using a glass Pasteur micropipette. The ablated embryos were subsequently incubated overnight at 37°C before visualization.

**Explant culture**

Chick embryos in HH10-14 stages were used. Embryos were positioned ventrally and one of the PSMs was divided into two halves - medial and lateral – parallel to the embryonic axis. In all explants, a second cut was made below the last formed somite and a third one immediately above the Hensen´s node (HN) to prevent the addition of new cells to the PSM over time. Tissues thus delimited and excised created two types of explants: a control one with an intact PSM, the axial structures and the medial portion of the contralateral PSM and an experimental explant containing only the correspondent lateral PSM portion. Explants were cultured individually for 9 hours, in a dorsal position, as previously described (Palmeirim et al., 1997). Similar results were obtained in all experimental conditions tested whether the control/experimental explants were the left or right one. Number of somites formed was assessed after clearing the explants in formamide.

Rescue of somite formation

For the incubations with ectopic notochord, the explants were delimited as described above but a notochord from a stage-matched embryo was juxtaposed to the L-PSM portion of the experimental explants. The notochord was enzymatically removed from the donor embryo using pancreatin (GIBCO) from the same A-P level considered for explant delimitation, i.e., from the last formed somite until the Hensen's node.

In the Shh supplementation experiments, experimental explants were cultured for 9h in medium containing 4µg/ml of Shh (kind gift of Sebastian Pons) while the controls were incubated in PBS / 10% glycerol supplemented medium.

**Immunohistochemistry**

In order to detect quail cells within the chimeric embryos, immunohistochemistry was carried out both in whole-mount embryos and in paraffin sections. To detect the quail's highly condensed heterochromatin, the monoclonal Quail Cell Perinuclear (QCPN) antibody (Developmental Studies Hybridoma Bank) was used, followed by a HRP-conjugated anti-mouse IgG antibody

(Southern Biotechnology) and diaminobenzidine tetrahydrochloride (DAB) revelation to evidence quail nuclei, as previously described (Charrier et al., 1999).

Whole-mount immunochemistry was performed according to the previously described procedure (Creuzet et al., 2002). For the immunohistochemistry in histological sections, the chimeras were fixed in modified Carnoy solution (100% ethanol, 37% formaldehyde solution and glacial acetic acid (6:3:1, V:V:V), embedded in paraffin, serially sectioned (5-7 mm) and processed as described (Charrier et al., 1999).

For cilia detection, stage HH12 embryos were fixed in 4% paraformaldehyde in 0.2 M phosphate buffer (PB) with 1 M CaCl2 and 4% sucrose overnight at 4°C. Embryos were then incubated in 0.12 M PB with 15% sucrose overnight at 4°C and finally in 0.12 M PB, 15% sucrose and 7.5% gelatin for 1 hr at 37°C. Processed embryos were then frozen in liquid nitrogen chilled isopentane, and stored at -80°C until sectioned. Transversal sections of 10 μm were made using Microm HM325 on SuperFrost Plus (Menzel-Glaser) slides. Sections were permeabilized for 20 min in PBS with 0.2% Triton X-100 and blocked for 30 min with 10% normal goat serum in PBS containing 1% bovine serum albumin. The primary anti-acetylated α-tubulin antibody (kind gift of Leonor Saúde) was diluted at 1:400 in the blocking solution and incubated overnight at 4°C. In the following day, sections were washed in PBS and incubated with Alexa Fluor 488-conjugated anti-mouse IgG, all F(ab')2 fragments (Molecular Probes) diluted 1:1000 in blocking solution, followed by PBS washes. Slides were stained with 4',6-Diamidino-2-phenylindole (DAPI, Sigma), mounted in Vectashield (Vector Laboratories) and sealed after cover-slipping.

**Statistical analysis**

Quantitative data are presented as mean value ± SD and were analyzed using the SigmaStat3.5 software (Systat software, Inc). For the number of somites formed in the different explants culture conditions, an ANOVA on Ranks analysis was performed.

## Results

### Segmentation of determined lateral PSM requires a medial-derived diffusible signal

Previous work from our group showed that lateral PSM cells are not able to segment in the absence of the medial-most portion, whereas M-PSM undergo normal somite formation after L-PSM ablation (Freitas et al., 2001). Moreover, expression of the segmentation molecular markers *delta1* (*dll1*), *paraxis*, *hairy1, hairy2* and *lunatic fringe* (*lfng*) was drastically down-regulated in L-PSMs deprived from their medial counterpart but remained normal in isolated M-PSMs. This

study showed that isolated L-PSM located above the determination front is unable to segment, suggesting that determined M-PSM cells are responsible for instructing and/or recruiting more lateral ones for somite formation. To understand the molecular nature of the medial-derived signal responsible for this effect, several experiments were carried out. To test whether this is achieved by a diffusible molecule, we performed *in ovo* microsurgery in HH12-14 stage chick embryos, inserting either a permeable or an impermeable barrier between the M- and L- portions of one PSM, while the counterlateral PSM remained intact (Fig. 1A). An impermeable barrier (tantalum foil) was used to prevent the exchange of diffusible signals between M- and L- PSM halves while the use of a permeable Millipore filter permitted the swap of diffusible substances between both PSM pieces. Operated embryos were re-incubated for 3 to 6 hours, which corresponds to the time required for the formation of 2 to 4 pairs of somites, respectively.

In embryos where an impermeable tantalum foil was inserted, the control side and the M-PSM underwent segmentation at the expected rate of one somite/90min (Fig. 1B; n=10). As expected, no somites were formed in the L-PSM portion. Note that the lack of segmentation in the L-PSM is not due to the reprogramming of the tissue to lateral plate mesoderm, as evidenced by performing *in situ* hybridization using the *bmp4* probe (Fig. 1B). In contrast, when a permeable barrier was used to separate M- and L- PSM, two contiguous rows of somites were observed in the experimental PSM, corresponding to segmentation of both M- and L- PSM compartments. The observed somites were formed at the same rate as the control side (Fig. 1C; n=11). Furthermore, both M- and L- PSM derived somites expressed *dll1* in their posterior half, indicating that a correct anterior-posterior (A-P) segregation occurred (Fig. 1C).

Taken together, these results indicate that the lateral portion of the PSM never truly becomes determined, as it is not capable of segmenting in the absence of its medial counterpart. Moreover, we show for the first time that the exchange of a diffusible signal from M- to L- PSM is required for recruitment of the L-PSM cells for somite formation.

**Shh is the diffusible molecule required for the recruitment of the anterior lateral PSM for segmentation**

An evident distinction between M- and L- portions of the PSM is that the M half is juxtaposed to the embryonic axial structures. Recent work from our lab has shown that notochord-derived Sonic hedgehog (Shh) is required for timely molecular oscillations and morphological somite formation. We also observed a graded expression of *patched1* (*ptch1*) along the M-L axis of determined PSM. These results lead us to postulate that Shh could be acting as a signaling gradient along the

PSM M-L axis, conferring M-PSM with the indispensable information for segmentation. To test if L-PSM recruitment for segmentation is mediated by Shh, we performed explant culture experiments. One of the chick embryo's PSM was longitudinally bisected, generating two types of explants (Fig. 2A): a control one containing an intact PSM (intact-PSM), the axial structures and the medial portion of the contralateral PSM (M-PSM) and an experimental explant, consisting of the lateral portion of the PSM alone (L-PSM). L-PSM explants thus generated were incubated either juxtaposed to an ectopic notochord or in the presence of soluble Shh diluted in the culture medium. Explants pairs were incubated separately for 9h and the number of somites formed during culture was assessed (Fig. 2B, C).

Explants containing the intact-PSM and the contralateral M-PSM portion always formed 5-6 somites, at the expected rate of one somite pair/90minutes (Fig. 2B, C). The M-PSM formed in average one somite less than the intact-PSM, probably due to the fact that this tissue tended to fold underneath the embryo midline structures. However, there were no significant differences in somite formation between intact-PSM and M-PSM (Fig. 2C). Therefore in the subsequent statistic analyses, the number of somites formed by the L-PSM was compared with those of the M-PSM. In the control conditions, the L-explant was unable to segment and only one somite was formed in very few cases (n=3/14) (Fig. 2B, C). When an ectopic notochord from a stage-matched embryo was juxtaposed to the L-explant (L+No), segmentation of the L-PSM occurred (n=13/14) (Fig. 2B, C). The same was observed in L-PSM explants cultured in the presence of soluble Shh (L+Shh, n=17/19) (Fig. 2B, C).

The results here presented further confirm that L-PSM is incapable of forming somites when isolated from the neighboring M-PSM domain. Furthermore, isolated L-PSM is able to segment in the presence of a Shh source, suggesting that Shh is the diffusible signal molecule traveling along the medial-lateral axis of the PSM to recruit L-PSM cells for segmentation.

**Prospective medial PSM cells are required for somite segmentation**

We next wondered when do PM cells acquire the intrinsinc information for segmentation and if this capacity would already be present in the presumptive PSM cells, i.e., if prospective medial (PM) and prospective lateral (PL) PSM cells present distinct commitment for segmentation. Previous results from our group have shown that in HH9$^{-}$ embryos, the PM-PSM territory is located deeply within the posterior open neural plate, in the most anterior part of the primitive streak immediately adjacent to the median pit (Freitas et al., 2001) (Fig. 3A). In the present work, we have localized the PL-PSM territory in HH9$^{-}$ embryos by performing quail/chick grafting experiments. A primitive streak fragment, extending over 100-150 μm rostrally from the caudal

limit of the posterior open neural plate, was replaced by the equivalent region of a stage-matched quail donor embryo (n=9). After 24h of incubation, quail cells within the chimeric embryo were localized by QCPN immunohistochemistry in histological sections. Donor cells were located in the lateral somite compartment, indicating that at this developmental stage L-PSM cells arise from the most caudal part of the open neural plate (Fig. 3A). These results show that within the prospective PSM territory, the most anterior regions (100-150 μm posteriorly to the median pit) give rise to more medial cells, while more posterior cells (100-150 rostrally from the caudal limit of the open neural plate) contribute to more lateral structures. This is in accordance with the generally accepted cellular organization within the primitive streak (Selleck and Stern, 1991; Schoenwolf et al., 1992; Psychoyos and Stern, 1996; Iimura et al., 2007).

We next analyzed the capacity of the PM- and PL- PSM cells for somite formation. We performed *in ovo* ablation of either the PL- or the PM- PSM territory in HH9$^{-}$ staged embryos and assessed the effect on segmentation 24h after the surgery. After PL-PSM territory excision, operated embryo segmentation proceeded normally, with the expected number of somites being formed (23-27 somites), indicating that this territory is not absolutely required for segmentation (Fig. 3B, n=6). In contrast, embryos in which the PM-PSM had been excised, presented no more than 18 somites (Fig. 3C, n=12). Note that at the moment of the surgery the embryo had 6 somite pairs and 12 prospective ones (somitomeres) were already located within the PSM (Meier, 1979), indicating that during the incubation period no PSM tissue with the ability to segment was formed. Even though somite formation was disrupted in these embryos, posterior elongation was still observed (white line in Fig. 3Cc') and PSM identity was not lost, as judged by *tbx6l* hybridization (Fig. 3Cc'').

Overall these results indicate that at the level of the prospective territory, PM- and PL- PSM cells are already different in what regards their commitment for segmentation. PM-PSM cells already contain the necessary information for somitogenesis whereas PL-PSM cells alone are not able to ensure somite formation.

**Prospective lateral PSM cells can be re-specified**

The PM- and PL- PSM territories of a chick HH9$^{-}$ embryo present distinct intrinsic information regarding somite formation. We next asked whether if at this developmental stage these territories are already definitively committed or if they can still be reverted. Heterotopic quail/chick grafts were performed between stage-matched embryos to generate chimeras with two PL territories by replacing the PM-PSM region of a chick host by the PL-PSM of a quail donor embryo (Fig. 4).

Operated embryos were then incubated for 24h and processed for QCPN immunostaining. Surprinsingly, these chimeras presented the expected number of somites, indicating that segmentation proceeded normally. After incubation, cells from the quail donor were located in the medial-most PSM and somites (Fig. 4, n=19). These results show that at this developmental stage, PL cells are not definitively commited and, when grafted in the PM-PSM territory, are able to acquire PM information. These results further suggest that PM-PSM is specified by a signalling coming environemental tissues.

**Median-pit signaling may specify prospective medial PSM cells**

The fact that PL-PSM cells acquire a medial fate when grafted into the PM domain suggests that PM cells are locally specified by neighboring cells. In HH9$^-$ embryos, the PM region is localized 100-150µm immediately below the median pit, which at this developmental stage corresponds to the early Hensen's node (HN), an important signaling center during chick development (reviewed in Charrier et al., 2005). Therefore, median pit signaling could be responsible for PM instruction. Since we previously show that Shh signaling is involved in recruiting the determined lateral cells in the anterior PSM, we analyzed the expression pattern of key signaling molecules of this pathway at the level of the median pit and prospective PSM territories of HH9$^-$ chick embryos (Fig. 5).

*In situ* hybridizations on paraffin sections revealed that *shh* is expressed in the notochord (no) and floor plate of the neural tube (fp), as expected (Fig. 5). At the median pit level, *shh* was detected in the axial-paraxial hinge region (aph), which corresponds to the junction of the presumptive midline axial structures and the paraxial mesoderm tissue (Charrier et al., 1999). Significant levels of *shh* are not detected in more posterior domains of the embryo (Fig. 5). These results are in accordance with previous observations (Charrier et al., 1999). Shh protein is known to act as a morphogen and, hence, is expected to be present as an A-P gradient along the PM- and PL- PSM domains. This could be the major difference between these territories. We also analyzed the expression of the Shh receptors, *ptch1/2*. *Ptch1* transcripts were clearly present in the floor plate (fp) and in the PSM, and were not significantly detected either in the aph or in the PM- or PL-PSM (Fig. 5). In the most anterior region analyzed, *ptch2* was found to be expressed in the notochord, neural tube (NT) and lateral plate (lp) (Fig. 5). At the median pit level, *ptch2* was strongly detected in the aph and more weakly in the neural plate (np). Moreover, *ptch2* was expressed in both the PM- and PL- PSM territories (Fig. 5). These results strongly suggest that Shh activity in the median pit and prospective PSM domains is mediated by this receptor.

## Discussion

### Medial and lateral PSM cells are differently committed for segmentation

Medial and lateral portions of PSM are produced at distinct times by ingression through the primitive streak (Selleck and Stern, 1991; Schoenwolf et al., 1992; Psychoyos and Stern, 1996; Sawada and Aoyama, 1999; Freitas et al., 2001; Eloy-Trinquet and Nicolas, 2002; Iimura et al., 2007). Reflecting this distinct origin is the fact that both medial and lateral PSM compartments are different in what regards their ability to segment: medial cells from the anterior most PSM region are able to undergo both morphologic and molecular segmentation even when isolated from their lateral counterparts while determined L-PSM does not segment when separated from the medial PSM domain (Freitas et al., 2001). Moreover, they have different fates in the adult body - medial PSM originates the vertebral column, epaxial muscles, and the dermis of the back while the lateral portion contributes to the hypaxial muscles (Ordahl and Le Douarin, 1992; Olivera-Martinez et al., 2000). Thus, medial PSM cells have intrinsic information for segmentation and in fact are the ones that give rise to the truly segmented structures of the vertebrate body. In the present work, we show that prospective M- and L- PSM domains are already differently commitment for segmentation. Ablation of PM-PSM disrupted somite formation whereas in the absence of the PL-PSM embryos developed normally (Fig. 3B). This suggests that medial determined PSM acquires the required information to drive somitogenesis while still in the PM territory. Even though somite formation was disrupted after PM ablation, the embryo was still able to elongate along its A-P axis (Fig.3Cc'). This is in accordance with a recent report were a series of caudal laser ablations were performed in HH10-11 chick embryos demonstrating that the strongest effect in axis elongation was observed after bilateral deletion of the caudal PSM (Benazeraf et al., 2010).

In order to further characterize the PSM presumptive domains, we have performed heterotopic quail/chick grafts. When the quail PL territory was placed in the chick PM position, the expected number of somites was formed and the medial-most PSM and somites were clearly populated by the PL-grafted tissue (Fig. 4). Thus, cells that normally give rise to more lateral fates acquired a medial fate and migrated to more medial positions. These results indicate that at stage HH9$^-$ PL cells are not predetermined and can be re-specified when grafted into a PM level. The observation that PL cells are able to acquire a medial fate suggests that PM identity is specified by a signal from neighboring cells and that PL cells are able to respond to this signal when placed in the PM position.

**Shh is involved in the specification of prospective medial PSM identity**

We have previously shown that timely somite formation in the undetermined PSM requires notochord-derived Shh (Resende et al., 2010) and questioned if it could also be implicated in conferring the ability to drive somitogenesis to the PM cells. When analyzing the expression of some of the Shh pathway components in the prospective PSM territory, we observed that *shh* transcripts are present in the median pit and in the axial-paraxial hinge (Charrier et al., 1999), while its receptor *ptch2* could be detected at high levels in this region and at lower levels in both medial and lateral presumptive territories (Fig. 5). It has been shown that in the chick *ptch2* is a target of the Hh pathway and that its transcription is activated even in the presence of low Shh levels, constituting a good readout of the pathway activity (Pearse et al., 2001). In fact, we observe an equivalent regulation in our explant culture system: in the absence of the source of Shh notochord, *ptch2* expression is completely absent even after prolonged incubation with the detection reagents (Fig. S1). The *ptch1* receptor was not significantly detected either in the median pit or in the prospective territories (Fig. 5). We have previously shown that the Shh receptor *smo* is ubiquitously expressed in the chick embryo mesoderm (Resende et al., 2010). Taken together, these results suggest that the prospective PSM cells are able to respond to Shh signaling.

We therefore suggest that in the prospective PSM territory, a gradient of Shh signaling activity is established. Shh from the median pit diffuses along the presumptive territory and is able to bind Ptch2 in the PM domain, activating the downstream signaling cascade in these cells. In this way, a gradient of tissue differentiation dependent on Shh concentration is created along the A-P axis of the presumptive PSM territory, such as observed in the neural tube and in the limb bud (Riddle et al., 1993; Yang et al., 1997; Briscoe et al., 2001): more anterior cells are exposed to high Shh levels and thus acquire a medial identity and progressively lower levels of Shh reaching the more posterior cells induce a lateral fate. Because PL cells express *ptch2* they can be converted into medial cells when exposed to elevated Shh concentrations.

**Shh mediates the recruitment of anterior lateral PSM cells for somite formation**

Previous results from our group showing that L- and M- PSM compartments possess distinct intrinsic information for somite formation and molecular segmentation suggest that a signal from M cells could be recruiting lateral ones for somite formation (Freitas et al., 2001). In the present work, we show that L-PSM cells are recruited to integrate a somite by a diffusible signal emanating from the M-PSM portion. Because Shh is implicated in the regulation of somite formation (Resende et al., 2010) and seems to be involved in PM–PSM specification (this work), this diffusible signaling molecule constitutes a good candidate to explain those observations.

Indeed, somite formation in isolated L-PSMs was recovered when explants were incubated in the presence of a Shh source, strongly suggesting that Shh is the diffusible signal emanating from the M- to the L- PSM compartments to induce somite formation. The medial-most PSM cells express *ptch1/2* and *smo* genes (Resende et al., 2010), enabling them to respond to Shh from the midline structures. Moreover, a careful analysis of the Ptch receptors' expression in the M-PSM shows a clear A-P gradient of *ptch1* expression, being that at the level of the next forming somite (S0, according to Pourquie and Tam, 2001) its expression spreads across the M-L PSM axis (Resende et al., 2010; Fig. S2) while in the prospective somites it is progressively restricted to the M territory. *Ptch2* does not present such graded expression, indicating that in the anterior PSM Shh probably exerts its effects mainly through Ptch1 receptor. Since *ptch1* is a direct target of the Shh pathway (Marigo et al., 1996; Marigo and Tabin, 1996), its increased expression in S0 suggests that these cells are actively responding to Shh signaling. This is further confirmed by the observation that the expression of the Shh transcriptional effectors *gli1*, *gli2* and *gli3* are absent from the PSM but are activated in this region (Borycki et al., 2000; Resende et al., 2010).

Taken together, these results lead us to propose the following model (Fig. 6): Shh emanating from the notochord binds Ptch receptors present in the M-PSM, triggering a signaling cascade in these cells. The ligand is further diffused across the PSM to reach its most lateral domain and a gradient of activity is formed along the M-L PSM axis. Dispatched1, protein required for long-range Shh signaling (Kawakami et al., 2002; Ma et al., 2002), may be involved in the propagation of the Shh signal (ongoing work). Primary cilium seems to play an important role in vertebrate Hh signaling (reviewed in Goetz and Anderson, 2010) and preliminary immunostaining results using anti-acetylated α-tubulin antibody, which recognizes stabilized microtubules, suggests the existence of cilia in the PSM cells (Fig. S3). Thus, M-PSM cells seem to have all the components necessary for proper Shh signaling. Indeed, M-PSM cells are probably actively implicated in the sharpening of the M-L Shh gradient since it has been shown that the regulatory feedback mechanisms of the pathway elicited by the responding cells are required for gradient spreading (Dessaud et al., 2007 and reviewed in Ribes and Briscoe, 2009). Thus, activation of the pathway in the M-PSM cells is required for lateral cell recruitment. Physical separation of the medial and lateral PSM domains disrupts the ability of medial cells to recruit lateral ones and thus no somites are formed by the lateral PSM cells. This ability is recovered upon exposure to Shh ligand. These cells are located above the determination front and thus are considered to be committed to integrate a somite (Dubrulle et al., 2001). However, our results suggest that lateral cells never truly become

determined and require additional Shh signaling to segment. The determination front may thus play a role in making anterior PSM cells responsive to Shh signaling.

Shh has been shown to regulate several adhesion molecules, being responsible for inducing increased cell-cell adhesion and tissue compaction (Testaz et al., 2001; Jarov et al., 2003). Somite formation involves a mesenhymal-to-epithelial transition, which is accompanied by re-arrangements in the extracellular matrix components. Moreover, it has been shown that PSM epithelialization initiates from the M-PSM cells that recruit more lateral ones to incorporate the somite, until the somite finally pulls apart from the PSM (Martins et al., 2009). Thus, we propose that Shh mediates the recruitment of more lateral cells by the medial PSM.

Besides the absence of somites, isolated L-PSM progressively loses the expression of *dll1* and the molecular clock gene *hairy1*, *hairy2* and *lfng* (Freitas et al., 2001; unpublished data). This suggests that a medial-to-lateral signal is also required for maintenance of the PSM molecular expression. In fact, in the absence of Shh, molecular clock gene expression is clearly perturbed and the clock pace is severely decreased (Resende et al., 2010), suggesting that Shh can be involved in synchronizing molecular expression along the M-L axis.

The above presented model points to a graded Shh activity along the PSM, similarly to what is observed in the limb bud and neural tube patterning (Riddle et al., 1993; Yang et al., 1997; Briscoe et al., 2001), which can be observed already in the prospective PSM territory. The proposed model explains our previous observation that in the absence of Shh by notochord ablation determined somites always form (Resende et al., 2010). In these experiments, the medial-most cells have been previously in contact with the Shh source, activating the molecular machinery required for somite formation. After Shh depletion, these cells are still able to propagate the Shh signaling across the M-L PSM and recruit more lateral ones. Accordingly, increasing concentrations of cyclopamine induced the formation of progressively less somites, suggesting that the effect of Shh is dose dependent. Moreover, in the absence of the notochord, the somites formed in a delayed fashion were progressively smaller along the M-L axis (Resende et al., 2010).

Taken together, the results here presented implicate Shh pathway in medial PSM specification and recruitment of lateral PSM cells for somite formation. This seems to be achieved by graded Shh activity along the A-P presumptive PSM and M-L determined PSM regions. With this work, we further implicate Shh signaling in the complex regulatory network operating during vertebrate embryo somitogenesis.

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**Figure Legends**

**Figure 1. A diffusible signal traveling from M-PSM to L-PSM cells is required for lateral PSM segmentation. (A)** Schematic representation of the microsurgical procedure in which a physical barrier (red) was inserted *in ovo* in one of the embryo's PSMs to separate medial (M) and lateral (L) presomitic mesoderm (PSM) compartments. SI, last formed somite. **(B, C)** Operated embryos in which either an impermeable tantalum foil barrier (B) or a Millipore permeable filter (C) was inserted between M- and L- PSM compartments. Embryos were incubated for 3 and 6h, respectively, and processed for i*n situ* hybridisation for lateral plate marker *bmp4* (B) and caudal somite marker *delta1* (C).

**Figure 2. Notochord-derived Shh is the diffusible molecule responsible for L-PSM recruitment for segmentation. (A)** Schematic representation of explant delimitation and excision. Two types of explants were generated: a control containing an intact PSM (I), the axial structures and the medial portion of the contralateral PSM (M-PSM, M) and an experimental explant, consisting of the lateral portion of the PSM only (L-PSM, L). Explant pairs were incubated separately for 9h. PSM, presomitic mesoderm; SI, last formed somite. **(B)** Representative cases of the different experimental conditions: explants delimited as described in (A) (control), explants to which an ectopic notochord was juxtaposed to the L-explant (L + No) and pairs in which the L-explant was incubated in the presence of soluble Sonic hedgehog (Shh) (L + Shh). White asterisks indicate somites formed during incubation (New so); I, intact PSM. **(C)** Graphical representation of the mean number of somites formed *de novo* in the different experimental conditions. Data are mean ± SD. *$p < 0.001$ *vs* control intact-PSM, control M-PSM, L+No L-PSM and L+Shh L-PSM. ns, not statistically significant.

**Figure 3. Cells located within the PM-PSM territory already posses the essential information for somitogenesis. (A)** Schematic representation of a 6-somite stage chick embryo in which a fragment located 100-150 µm rostrally from the caudal limit of the open neural plate was replaced by a quail equivalent piece (red square) to identify the prospective lateral (PL) PSM territory. The localization of the prospective medial (PM) PSM domain is also indicated. Immunostaining in sections with QCPN shows the localization of the quail descendent cells in lateral somites (a). PSM, presomitic mesoderm; SI, last formed somite. **(B)** Dorsal view of a PL-PSM excised embryo at the moment of excision (b, t0) and upon 24 hours of incubation (b', t24). **(C)** Dorsal view of an embryo following excision of the PM-PSM territory (c, t0), after 24 hours of incubation (c', t24) and after *in situ* hybridization with *tbx6l* probe (c''). The white line evidences embryo elongation during culture. Rostral is towards the top.

**Figure 4. PM-PSM specification is dependent on an environmental signal. (A)** Quail/chick chimeras containing two PL territories were generated by replacing the PM-PSM region of a chick host with the PL-PSM of a quail donor embryo (brown square). Representative result of whole-mount QCPN immunostaining after 24h incubation is shown. Rostral is towards the top. PSM, presomitic mesoderm; SI, last formed somite.

**Figure 5. PM-PSM cells express Shh signaling components.** HH9$^{-}$ chick embryos were processed for *in situ* hybridization in paraffin sections for the genes *sonic hedgehog* (*shh*), *patched1* (*ptch1*) and *patched2* (*ptch2*). Representative results for distinct anterior-posterior positions are shown: anteriorly to the open neural plate (a), at the axial-paraxial hinge (aph) level (b), PM-PSM (c) and PL-PSM (d). PSM, presomitic mesoderm; SI, last somite formed; no, notochord; fp, floor plate of the neural tube; NT, neural tube; lp, lateral plate; np, neural plate.

**Figure 6. Proposed model for the two-step somite formation-dependence on Shh signaling.** Schematic representation of the posterior portion of an embryo, depicting the presomitic mesoderm (PSM) and the last two formed somites (SI, SII). Prospective PSM territory and determined PSM tissue are indicated. The Sonic hedgehog (Shh) receptor *patched2* (*ptch2*) presents a graded anterior-posterior expression in the prospective PSM (green striped square), *ptch1* is expressed faintly along the entire medial PSM but its expression is higher in the anterior PSM (green triangle) while *smoothened* is ubiquitously expressed in the PSM (grey). Shh produced by the axial structures binds Ptch2 receptor in the prospective PSM territory activating the Shh pathway in this domain, which is presumably responsible for the specification of the

prospective medial (PM) PSM cells. In the anterior PSM, Shh activates its downstream targets in the medial PSM domain through binding to Ptch1. This signaling is further propagated across the medial-lateral PSM axis and is probably involved in the recruitment of lateral cells by medial ones to integrate a somite (dotted line).

**Figure S1. *Patched2* transcription is regulated by Shh.** Representative result of *patched2* expression in chick embryo explants cultured for 4.5h with (No+) or without (No-) the notochord. Expression of *patched2* is downregulated in the absence of the notochord, source of Shh, indicating that this gene is directly regulated by the Shh signaling pathway.

**Figure S2. *Patched1/2* expression in the medial PSM.** Representative results of *patched1* and *patched2* expression in HH13 and HH12 whole embryo, respectively. White arrowed indicates the last formed somite. White asterisks along the anterior PSM of the HH13 embryo evidence the concomitant graded anterior-posterior and medial-lateral expression of *patched1* observed in this region. Graded expression is not as significant for *patched2* transcripts.

**Figure S3. PSM cells present cilia.** Stage Hh12 chick embryos were sectioned and immunostaining was performed at the presomitic mesoderm (PSM) level for acetylated α-tubulin (green) and DNA (Dapi, blue), as indicated. A representative result is presented, indicating the presence of few cilia in the PSM (white arrows). The region within the white square is presented at higher magnification (4x zoom). The medial-lateral and anterior-posterior orientation is indicated. NT, neural tube.

**References**

Andrade, R. P., Palmeirim, I. and Bajanca, F. (2007) 'Molecular clocks underlying vertebrate embryo segmentation: A 10-year-old hairy-go-round', *Birth Defects Res C Embryo Today* 81(2): 65-83.

Aulehla, A., Wehrle, C., Brand-Saberi, B., Kemler, R., Gossler, A., Kanzler, B. and Herrmann, B. G. (2003) 'Wnt3a plays a major role in the segmentation clock controlling somitogenesis', *Dev Cell* 4(3): 395-406.

Benazeraf, B., Francois, P., Baker, R. E., Denans, N., Little, C. D. and Pourquie, O. (2010) 'A random cell motility gradient downstream of FGF controls elongation of an amniote embryo', *Nature* 466(7303): 248-52.

Borycki, A.-G., Brown, A. M. C. and Emerson, J. C. P. (2000) 'Shh and Wnt signaling pathways converge to control Gli gene activation in avian somite', *Development* 127: 2075-2087.

Briscoe, J., Chen, Y., Jessell, T. M. and Struhl, G. (2001) 'A Hedgehog-Insensitive Form of Patched Provides Evidence for Direct Long-Range Morphogen Activity of Sonic Hedgehog in the Neural Tube', *Molecular Cell* 7: 1279-1291.

Cairns, D. M., Sato, M. E., Lee, P. G., Lassar, A. B. and Zeng, L. (2008) 'A gradient of Shh establishes mutually repressing somitic cell fates induced by Nkx3.2 and Pax3', *Dev Biol* 323(2): 152-65.

Charrier, J. B., Catala, M., Lapointe, F., Le-Douarin, N. and Teillet, M. A. (2005) 'Cellular dynamics and molecular control of the development of organizer-derived cells in quail-chick chimeras', *Int J Dev Biol* 49(2-3): 181-91.

Charrier, J. B., Teillet, M. A., Lapointe, F. and Le Douarin, N. M. (1999) 'Defining subregions of Hensen's node essential for caudalward movement, midline development and cell survival', *Development* 126(21): 4771-83.

Chiang, C., Litingtung, Y., Lee, E., Young, K. E., Corden, J. L., Westphal, H. and Beachy, P. A. (1996) 'Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function', *Nature* 383(6599): 407-13.

Christ, B. and Ordahl, C. P. (1995) 'Early stages of chick somite development', *Anat.Embryol.(Berl)* 191(5): 381-396.

Creuzet, S., Couly, G., Vincent, C. and Le Douarin, N. M. (2002) 'Negative effect of Hox gene expression on the development of the neural crest-derived facial skeleton', *Development* 129(18): 4301-13.

Dequeant, M. L., Glynn, E., Gaudenz, K., Wahl, M., Chen, J., Mushegian, A. and Pourquie, O. (2006) 'A complex oscillating network of signaling genes underlies the mouse segmentation clock', *SCIENCE* 314(5805): 1595-8.

Diez del Corral, R., Olivera-Martinez, I., Goriely, A., Gale, E., Maden, M. and Storey, K. (2003) 'Opposing FGF and retinoid pathways control ventral neural pattern, neuronal differentiation, and segmentation during body axis extension', *Neuron* 40(1): 65-79.

Dubrulle, J., McGrew, M. J. and Pourquie, O. (2001) 'FGF signaling controls somite boundary position and regulates segmentation clock control of spatiotemporal Hox gene activation', *Cell* 106(2): 219-232.

Eloy-Trinquet, S. and Nicolas, J. F. (2002) 'Cell coherence during production of the presomitic mesoderm and somitogenesis in the mouse embryo', *Development* 129(15): 3609-3619.

Fan, C.-M. and Tessier-Lavignet, M. (1994) 'Patterning of mammalian somites by surface ectoderm and notochord: evidence for sclerotome induction by a hedgehog homolog', *Cell* 79(7): 1175-8.

Francis, P. H., Richardson, M. K., Brickell, P. M. and Tickle, C. (1994) 'Bone morphogenetic proteins and a signalling pathway that controls patterning in the developing chick limb', *Development* 120(1): 209-18.

Freitas, C., Rodrigues, S., Charrier, J. B., Teillet, M. A. and Palmeirim, I. (2001) 'Evidence for medial/lateral specification and positional information within the presomitic mesoderm', *Development* 128(24): 5139-47.

Goetz, S. C. and Anderson, K. V. (2010) 'The primary cilium: a signalling centre during vertebrate development', *Nat Rev Genet* 11(5): 331-44.

Hamburger, V. and Hamilton, H. L. (1992) 'A series of normal stages in the development of the chick embryo. 1951', *Dev.Dyn.* 195(4): 231-272.

Henrique, D., Adam, J., Myat, A., Chitnis, A., Lewis, J. and Ish-Horowicz, D. (1995) 'Expression of a Delta homologue in prospective neurons in the chick', *Nature* 375(6534): 787-790.

Iimura, T., Yang, X., Weijer, C. J. and Pourquie, O. (2007) 'Dual mode of paraxial mesoderm formation during chick gastrulation', *Proc Natl Acad Sci U S A* 104(8): 2744-9.

Incardona, J. P., Gaffield, W., Kapur, R. P. and Roelink, H. (1998) 'The teratogenic Veratrum alkaloid cyclopamine inhibits sonic hedgehog signal transduction', *Development* 125(18): 3553-62.

Jarov, A., Williams, K. P., Ling, L. E., Koteliansky, V. E., Duband, J. L. and Fournier-Thibault, C. (2003) 'A dual role for Sonic hedgehog in regulating adhesion and differentiation of neuroepithelial cells', *Dev.Biol.* 261(2): 520-536.

Kawakami, T., Kawcak, T., Li, Y. J., Zhang, W., Hu, Y. and Chuang, P. T. (2002) 'Mouse dispatched mutants fail to distribute hedgehog proteins and are defective in hedgehog signaling', *Development* 129(24): 5753-65.

Kulesa, P. M. and Fraser, S. E. (2002) 'Cell dynamics during somite boundary formation revealed by time-lapse analysis', *SCIENCE* 298(5595): 991-995.

Ma, Y., Erkner, A., Gong, R., Yao, S., Taipale, J., Basler, K. and Beachy, P. A. (2002) 'Hedgehog-mediated patterning of the mammalian embryo requires transporter-like function of dispatched', *Cell* 111(1): 63-75.

Marigo, V., Johnson, R. L., Vortkamp, A. and Tabin, C. J. (1996) 'Sonic hedgehog differentially regulates expression of GLI and GLI3 during limb development', *Dev Biol* 180(1): 273-83.

Marigo, V. and Tabin, C. J. (1996) 'Regulation of Patched by Sonic hedgehog in the developing neural tube', *Proc. Natl. Acad. Sci. USA* 93: 9346-9351.

Martins, G. G., Rifes, P., Amandio, R., Rodrigues, G., Palmeirim, I. and Thorsteinsdottir, S. (2009) 'Dynamic 3D cell rearrangements guided by a fibronectin matrix underlie somitogenesis', *PLoS One* 4(10): e7429.

Meier, S. (1979) 'Development of the chick embryo mesoblast. Formation of the embryonic axis and establishment of the metameric pattern', *Dev Biol* 73(1): 24-45.

Olivera-Martinez, I., Coltey, M., Dhouailly, D. and Pourquie, O. (2000) 'Mediolateral somitic origin of ribs and dermis determined by quail-chick chimeras', *Development* 127(21): 4611-7.

Ordahl, C. P. and Le Douarin, N. M. (1992) 'Two myogenic lineages within the developing somite', *Development* 114(2): 339-53.

Palmeirim, I., Henrique, D., Ish-Horowicz, D. and Pourquie, O. (1997) 'Avian hairy gene expression identifies a molecular clock linked to vertebrate segmentation and somitogenesis', *Cell* 91(5): 639-648.

Pearse, R. V., Vogan, K. J. and Tabin, C. J. (2001) 'Ptc1 and Ptc2 Transcripts Provide Distinct Readouts of Hedgehog Signaling Activity during Chick Embryogenesis', *Dev.Biol.* 239: 15-29.

Pourquie, O. and Tam, P. P. L. (2001) 'A nomenclature for prospective somites and phases of cyclic gene expression in the presomitic mesoderm', *Dev Cell* 1(5): 619-20.

Psychoyos, D. and Stern, C. D. (1996) 'Fates and migratory routes of primitive streak cells in the chick embryo', *Development* 122(5): 1523-34.

Resende, T. P., Ferreira, M., Teillet, M. A., Tavares, A. T., Andrade, R. P. and Palmeirim, I. (2010) 'Sonic hedgehog in temporal control of somite formation', *Proc Natl Acad Sci U S A* 107(29): 12907-12.

Ribes, V. and Briscoe, J. (2009) 'Establishing and interpreting graded Sonic Hedgehog signaling during vertebrate neural tube patterning: the role of negative feedback', *Cold Spring Harb Perspect Biol* 1(2): a002014.

Riddle, R. D., Johnson, R. L., Laufer, E. and Tabin, C. (1993) 'Sonic hedgehog mediates the polarizing activity of the ZPA', *Cell* 75(7): 1401-16.

Rong, P. M., Teillet, M.-A., Ziller, C. and Le Douarin, N. M. (1992) 'The neural tube/notochord complex is necessary for vertebral but not limb and body wall striated muscle differentiation', *Development* 115: 657-672.

Sawada, K. and Aoyama, H. (1999) 'Fate maps of the primitive streak in chick and quail embryo: ingression timing of progenitor cells of each rostro-caudal axial level of somites', *Int.J Dev.Biol.* 43(8): 809-815.

Schoenwolf, G. C., Garcia-Martinez, V. and Dias, M. S. (1992) 'Mesoderm movement and fate during avian gastrulation and neurulation', *Dev Dyn* 193(3): 235-48.

Selleck, M. A. and Stern, C. D. (1991) 'Fate mapping and cell lineage analysis of Hensen's node in the chick embryo', *Development* 112(2): 615-26.

Teillet, M.-A., LAPOINTE, F. and Le Douarin, N. M. (1998) 'The relationships between notochord and floor plate in vertebrate development revisited', *Proc. Natl. Acad. Sci. USA* 95 11733-11738.

Testaz, S., Jarov, A., Williams, K. P., Ling, L. E., Koteliansky, V. E., Fournier-Thibault, C. and Duband, J. L. (2001) 'Sonic hedgehog restricts adhesion and migration of neural crest cells independently of the Patched- Smoothened-Gli signaling pathway', *Proc.Natl.Acad.Sci.U.S.A* 98(22): 12521-12526.

Tozer, S., Bonnin, M. A., Relaix, F., Di Savino, S., Garcia-Villalba, P., Coumailleau, P. and Duprez, D. (2007) 'Involvement of vessels and PDGFB in muscle splitting during chick limb development', *Development* 134(14): 2579-91.

Varjosalo, M. and Taipale, J. (2008) 'Hedgehog: functions and mechanisms', *Genes Dev* 22(18): 2454-72.

Yang, Y., Drossopoulou, G., Chuang, P. T., Duprez, D., Marti, E., Bumcrot, D., Vargesson, N., Clarke, J., Niswander, L., McMahon, A. et al. (1997) 'Relationship between dose, distance and time in Sonic Hedgehog-mediated regulation of anteroposterior polarity in the chick limb', *Development* 124(21): 4393

**Figure 1**

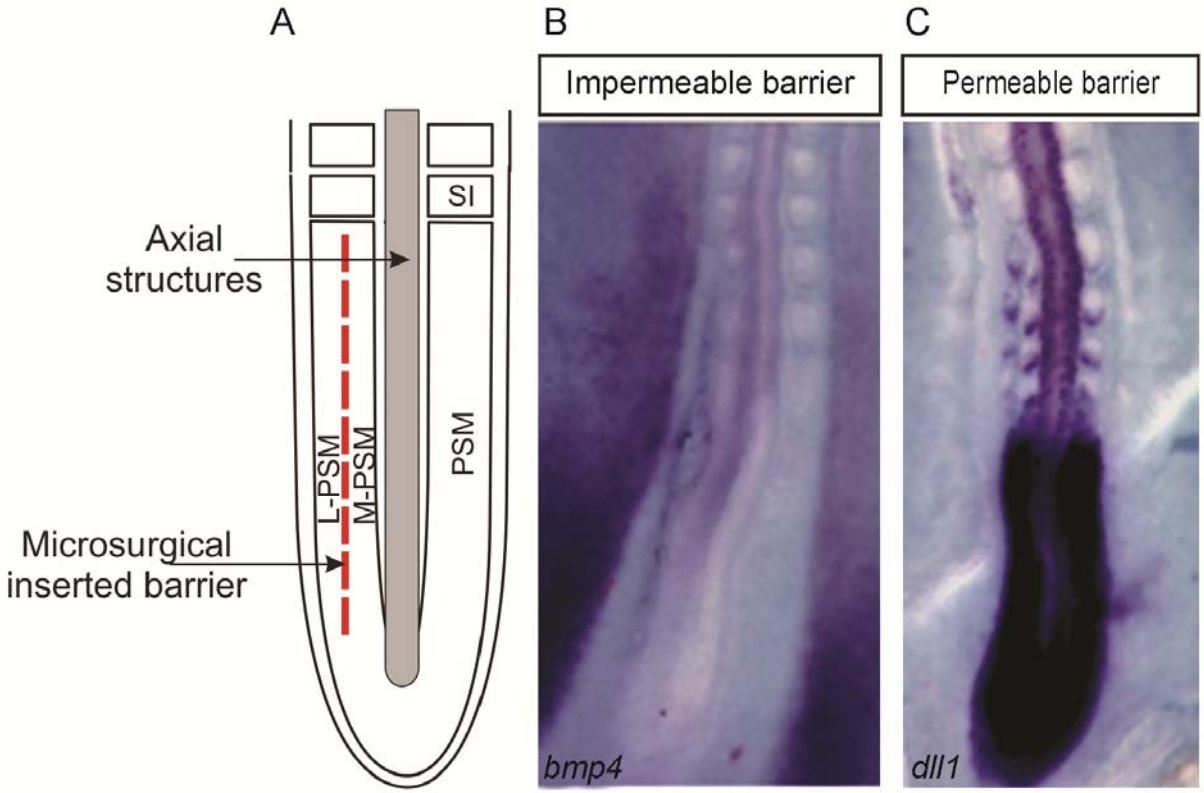

A
B
C
Impermeable barrier
Permeable barrier
SI
Axial structures
L-PSM
M-PSM
PSM
Microsurgical inserted barrier
bmp4
dll1

**Figure 2**

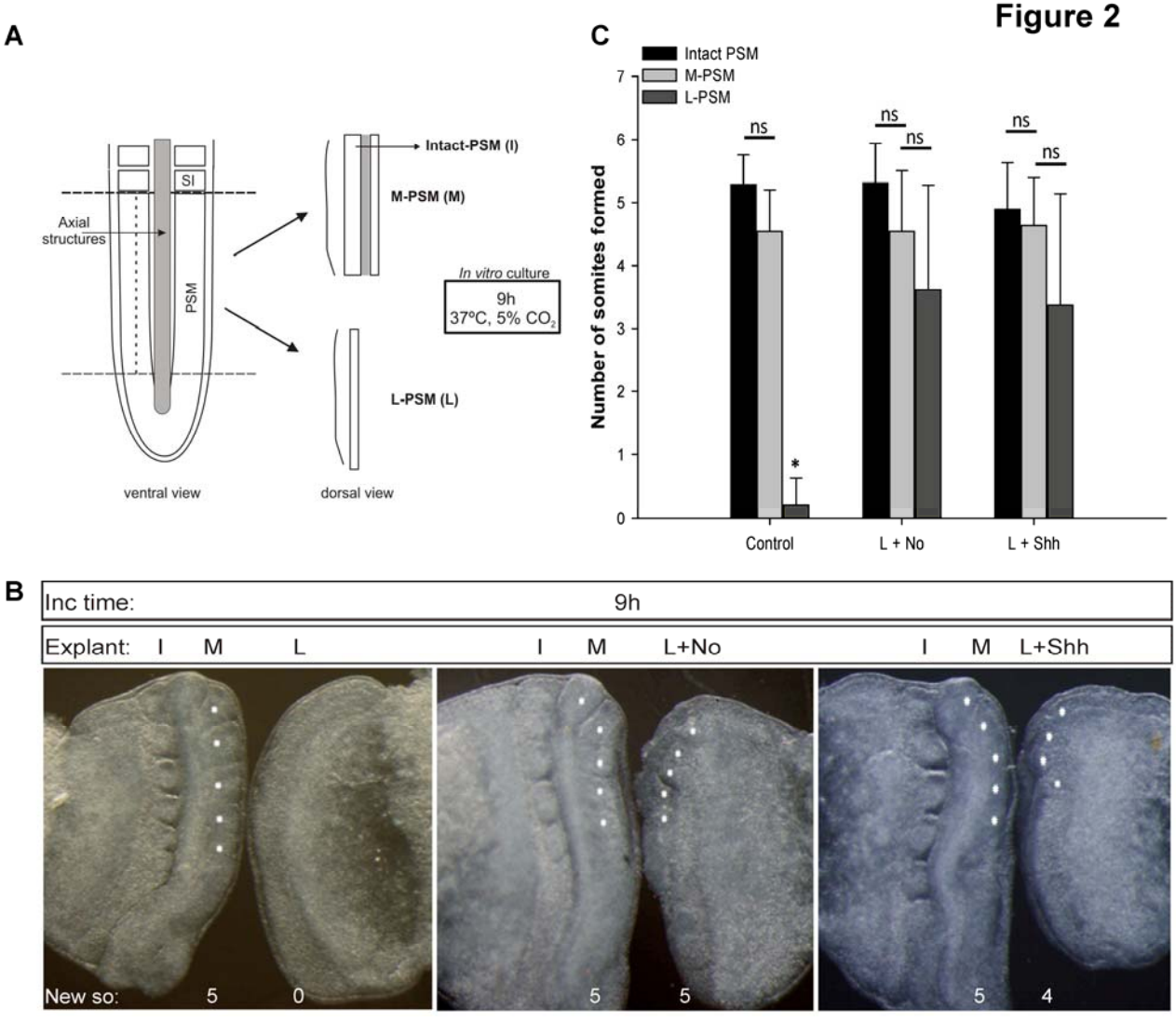

A
Intact-PSM (I)
M-PSM (M)
SI
Axial structures
PSM
In vitro culture
9h
37°C, 5% CO2
L-PSM (L)
ventral view
dorsal view
C
Intact PSM
M-PSM
L-PSM
Number of somites formed
ns
*
Control
L + No
L + Shh
B
Inc time: 9h
Explant: I M L I M L+No I M L+Shh
New so: 5 0 5 5 5 4

**Figure 3**

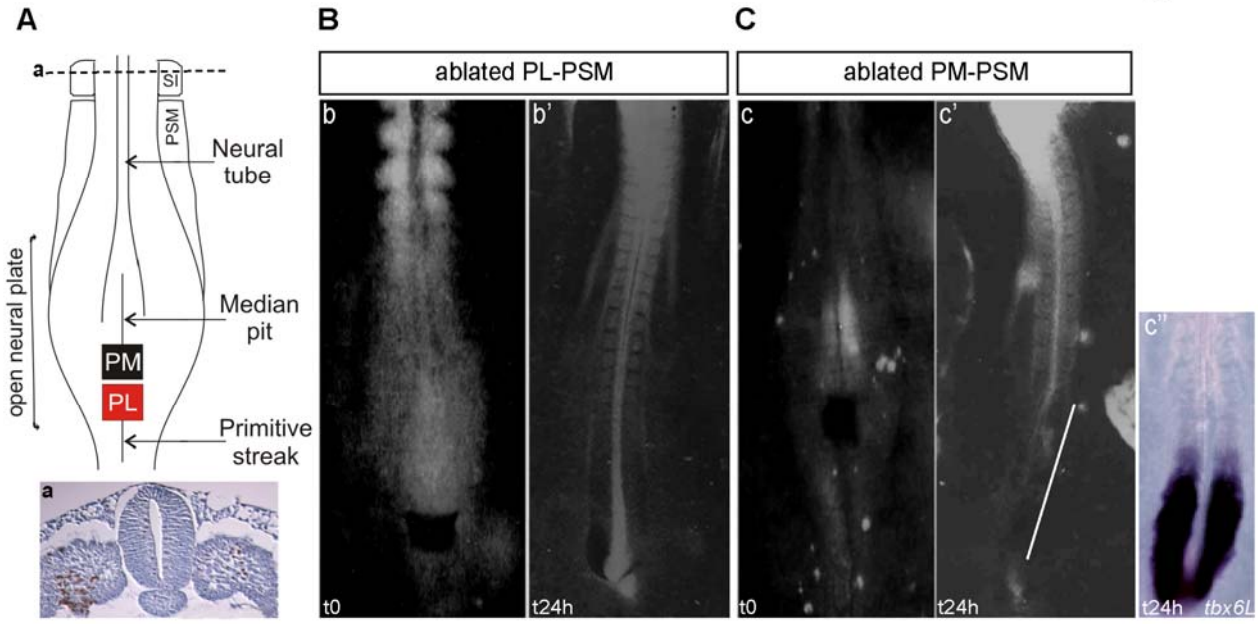

A
B
C
a
SI
PSM
Neural tube
Median pit
PM
PL
Primitive streak
open neural plate
a
ablated PL-PSM
ablated PM-PSM
b
b'
c
c'
c''
t0
t24h
t0
t24h
t24h tbx6L

**Figure 4**

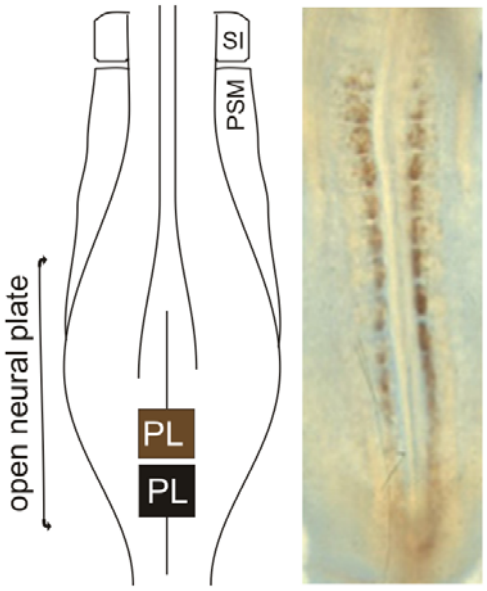


**Figure 5**

**Figure 6**

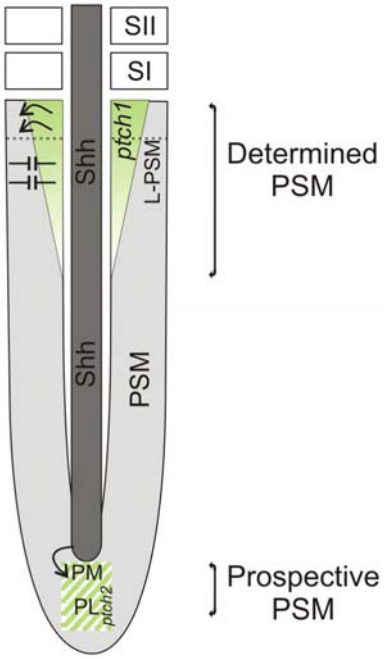

SII
SI
Shh
ptch1
L-PSM
Determined PSM
Shh
PSM
PM
PL
ptch2
Prospective PSM

**Figure S1**

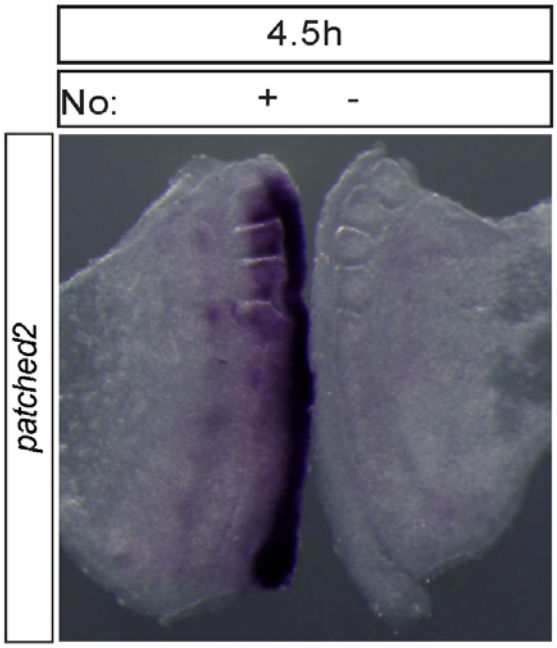
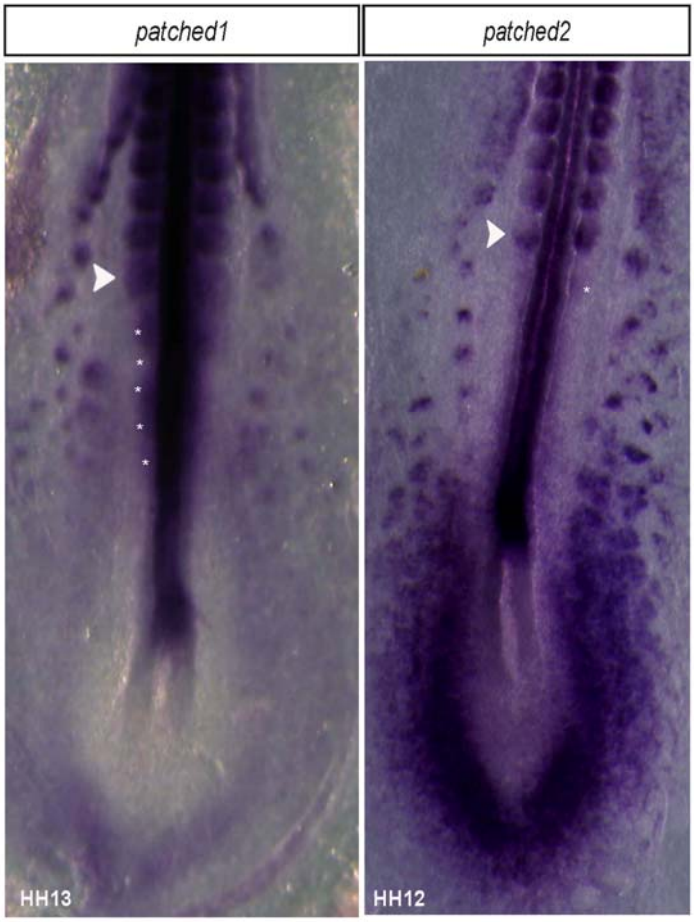


**Figure S2**



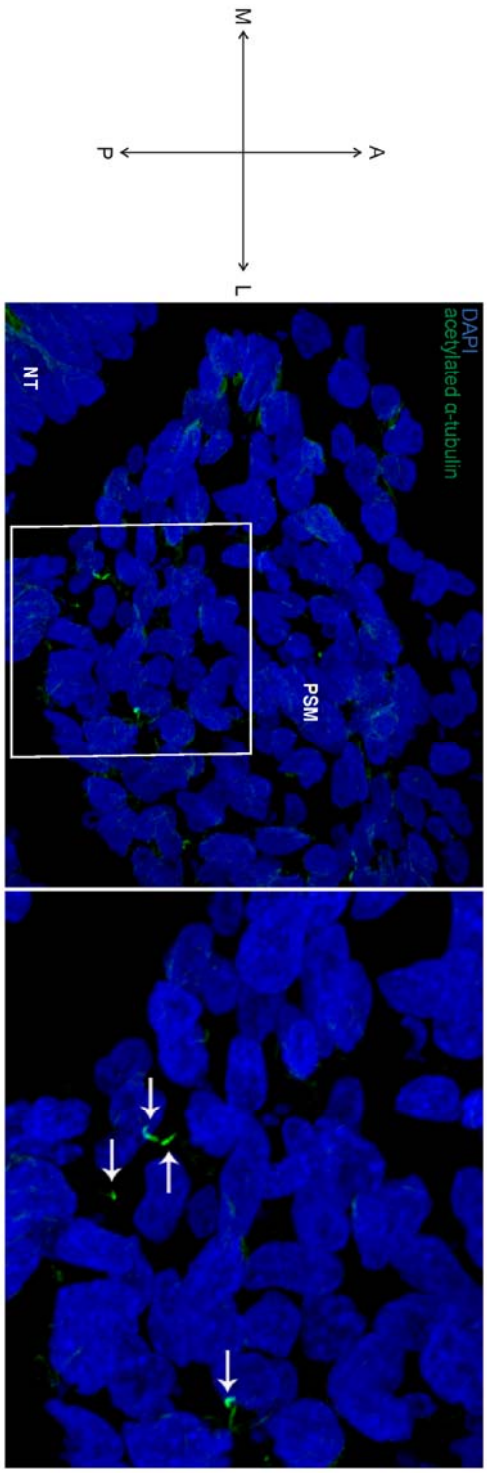



Figure S3

# CHAPTER4.

# GENERAL DISCUSSION

## 4. SONIC HEDGEHOG IN SOMITOGENESIS

The striking periodicity of somite formation observed in vertebrate embryos is now considered a model for understanding temporal control of Biological processes. The description of a molecular clock underlying somitogenesis has brought important insight into the regulatory mechanisms operating during this embryologic event. In the last years, the description of molecular interactions between distinct signaling pathways operating in concert to regulate timely somite formation has remarkably increased our knowledge on somitogenesis. However, it has also revealed the high complexity of this process.

Several attempts have been made to alter the periodic somite formation. At the experimental manipulation level, Packard and collaborators have performed several interesting analysis. By performing embryo explant culture these authors proposed that somite formation is independent of the neural tube and notochord (Packard and Jacobson, 1976; Packard, 1980). However, these explants were cultured for long periods (~14h) and the rate of somite formation during incubation was never assessed. Another work from the same group showed that grafting of a quail PSM into a chick embryo induced the progressive adjustment of somite formation to the host PSM (Packard et al., 1993). In these experiments, the somites formed by the quail PSM located above the determination front at the time of the graft were not aligned with those formed by the host's PSM. Somites from the undetermined PSM, however, were gradually aligned with those of the chick. This strongly suggested that a signal from the embryo midline structures was required to bilaterally adjust for somite formation.

The work presented throughout this thesis addresses the midline contribution to vertebrate embryo somitogenesis. We clearly demonstrated that notochord derived Shh is implicated in temporal control of somite formation and molecular clock oscillations. Moreover, we have established a role for Shh in PSM cell fate specification and somitic cell recruitment during segmentation. Overall, the results here presented provide concluding evidence that Shh signaling pathway is required for proper PSM segmentation, further clarifying the molecular regulations occurring in the PSM during somitogenesis.

### 4.1 SHH PATHWAY OPERATES IN THE PSM

Shh signaling pathway is classically associated with somite D-V differentiation but not with PSM segmentation. Although it has been shown that the expression of Shh downstream Gli

effectors is activated concomitantly with somite formation (Borycki et al., 2000), this has only been associated with an initiation of Shh responsiveness in newly formed somites, required for somite cell maintenance and differentiation (Rong et al., 1992; Fan and Tessier-Lavignet, 1994; Teillet et al., 1998). We have analyzed the expression of the pathway's membrane receptors and shown that *ptch1/2* are expressed in the medial PSM tissue and that *smo* is ubiquitously present in the PSM, creating the conditions for Shh signaling readout in this tissue. *In situ* hybridization on sections suggests the existence of a *ptch1* A-P expression gradient, with more transcripts being detected in the determined PSM. In this tissue, a graded expression of *ptch1* was also observed along the M-L axis – medial most PSM cells present high *ptch1* levels, which decrease towards the lateral region. A similar behavior was observed for *ptch2* along the PSM prospective A-P axis – this gene is highly expressed in the median pit and lower levels are detected in the PM and PL domains. This suggests that Shh signaling is active in the PSM, including its presumptive territory, and activation of the pathway is mediated by Ptch1 or Ptch2 receptors. Since primary cilium seems to play an important role in vertebrate Hh signaling, we have analyzed the PSM in search for cilia. Preliminary immunostaining results using anti-acetylated α-tubulin antibody, suggests the existence of cilia in the PSM cells. However, a more detailed analysis using markers for the basal body (γ-tubulin) is required. Overall, the data here presented indicate that both the anterior PSM and the P-PSM posses the molecular players required for Shh signal transduction.

## 4.2 TIMELY SOMITE FORMATION IS REGULATED BY SHH SIGNALING

We evaluated the rate of somite formation over time in explants cultured with or without the notochord. Our results indicate that the anterior third of the PSM, which corresponds to the determined PSM (Dubrulle et al., 2001), does not require the presence of the notochord to segment at the expected rate of one somite/90min. This is in accordance with Packard's grafting results (Packard and Meier, 1983) and suggests that this tissue is also temporally determined. However, somite formation from the undetermined PSM is severely delayed in the absence of the notochord, although not impaired. In fact, the formation of the first three undetermined somites is resumed after 9h-12h of incubation, suggesting that the system is capable of overcoming the notochord absence. We further show that Shh chemical inhibition renders similar results, indicating that notochord-mediated Shh is necessary for timely somite formation. Since *ptch1* positively regulates the Hh pathway (Chen and Struhl, 1996; Jeong and McMahon, 2005), increased expression of this gene in the medial-most determined PSM indicates that these cells are actively responding to Shh signaling, which further supports our observations.

Previous work from our group has shown that in the determined PSM, medial and lateral domains present distinct intrinsic abilities regarding somite formation and molecular segmentation (Freitas et al., 2001). In fact, the determined L-PSM does not segment in the absence of the M-PSM, suggesting that lateral cells never truly become committed and that medial cells recruit lateral ones for somite formation. In accordance, we show that this recruitment is mediated by a diffusible signal traveling from the M- to the L- anterior PSM. Moreover, the M-PSM function can be replaced by an ectopic notochord or by delivering exogenous Shh to the L-PSM explant, suggesting that in the determined PSM Shh is required for M-L somite cell recruitment. We thus propose that Shh emanating from the midline structures activates the downstream signaling cascade in the M-PSM cells, through Ptch1, establishing an M-L gradient of Shh activity in the PSM, which is required for lateral cells recruitment into the forming somite. Accordingly, somites formed in the absence of the notochord are smaller along their M-L axis, suggesting that in these experimental conditions M-PSM cells possess lower levels of Shh signaling, generating a steaper M-L gradient which culminates in fewer cells being recruited to integrate the forming somite. It has been shown that cell re-arrangements for somite formation start in the M-PSM domain (Martins et al., 2009). These cells are the first to become epithelial and are then thought to progressively recruit more lateraly located cells to incorporate the somite, until it finally pulls apart from the PSM. Shh has been shown to regulate several adhesion molecules required for cell movement and epithelialization (Testaz et al., 2001; Jarov et al., 2003) and can therefore be involved in this morphological event. Besides the negative feedback loops which could be cell-autonomously regulating the establishment of the gradient along the M-L axis (reviewed in Dessaud et al., 2008; Ribes and Briscoe, 2009), we foresee the existence of an opposed lateral-to-medial signal from the lateral plate mesoderm counteracting the Shh gradient. A good candidate is BMP signaling, which has been shown to counteract Shh during somite differentiation (Reshef et al., 1998 and reviewed in Stockdale et al., 2000). *Bmp4* expression in explants without the M-PSM is still confined to the lateral plate, indicating that the L-PSM tissue is not re-specified (Freitas et al., 2001 and our observations). Another possibility is the transcription factor *pax3*, which is expressed in the lateral plate (data not shown) and has been shown to be regulated by Shh in a dose-dependent manner: in the differentiating somite, low Shh levels reaching the dermamyotome induce *pax3* while high Shh levels induce *nkx3.2* in the sclerotome, inhibiting *pax3* in this compartment (Chiang et al., 1996; Cairns et al., 2008). At the PSM level a similar regulation might occur: graded Shh activity along the M-L PSM could be counteracted by *pax3* or other

genes from the lateral plate, in order to establish the proper graded signaling activity required for somite segmentation.

## 4.3 SHH DOSE- AND TIME- DEPENDENT RESPONSE IN PSM

In the last years, it has been proposed that Shh dependent specification relies not only on the ligand concentration but also on the time period of exposure (Dessaud et al., 2007; Dessaud et al., 2010). Thus, changing the concentration or the duration of Shh has an equivalent effect on the intracellular signaling (Stamataki et al., 2005). Grafting experiments indicate that at developmental stage HH9$^-$, the PL-PSM domain can be re-specified if grafted into a PM position. Analysis of Shh components expression at the prospective PSM region indicates that PM cells are exposed to higher Shh doses than the PL domain, and this presumably specifies their medial fate. Besides being under the influence of higher Shh doses, the M-PSM cells are also exposed to this signaling pathway for a longer period of time since they are adjacent to the midline structures and express *ptch1*, even if at lower levels when located behind the determination front. In this tissue, it is plausible that lateral cells do not receive Shh signaling. With embryo elongation, PSM cells are progressively displaced to a more anterior region and when located in the anterior PSM, M-cells are once more subjected to high Shh signaling. It seems likely that M-PSM specification relies on both high Shh concentration and exposure period. Our observations fit well with this "temporal adaptation" model (Dessaud et al., 2007; Dessaud et al., 2010). At the time of explant delimitation, determined and undetermined PSM tissue had been exposed to Shh for different time periods and to distinct Shh doses. The anterior PSM was at the moment of excision under the influence of high Shh and actively responding to this signaling, as judged by increased *ptch1* expression. Thus, they already possessed intrinsic information that enabled them to recruit lateral cells even in the absence of continuous Shh supply. On the other hand, medial cells located behind the determination front at the time of explant delimitation had "seen" Shh for a shorter time period and at the excision moment were not yet actively responding to it (they present lower *ptch1* expression). Therefore, these cells do not posses intrinsic information to recruit lateral ones for timely segmentation. This is further supported by the fact that Shh chemical inhibition presents a dose-dependent effect on determined somite formation: increasingly higher cyclopamine doses results in the formation of progressively less somites. Moreover, the highest cyclopamine concentration tested almost completely abrogated somite formation - only one somite was formed after 9h of incubation. Cyclopamine interferes with the Shh pathway through direct inhibition of Smo receptor activity, thus interrupting the signaling cascade even in the presence of Ptch

(Incardona et al., 1998). So, anterior M-PSM cells expressing *ptch1* are not able to recruit lateral cells after Shh pathway blockade. A dose-dependent Shh effect was also observed in the rescue experiments: addition of Shh-expressing cells was only able to recover somite formation and gene expression when big clumps of cells were juxtaposed to the explant's neural tube. This observation further indicates that M-PSM cells require high Shh concentrations for proper segmentation. To confirm that M-PSM cells are specified by longer exposure to Shh, we intend to inhibit Shh signaling in the PM-PSM and verify if somite formation occurs. Shh inhibition in the PM-PSM territory can be achieved either by placing a bead soaked in cyclopamine in this region or electroporating the PM-PSM with the Ptch1$^{\Delta loop2}$ construct, which codifies a deleted form of Ptch1 that does not bind Shh but is able to bind and inhibit Smo (Briscoe et al., 2001). If Shh is indeed the signaling molecule responsible for medial fate specification, we expect to obtain a similar result to the one observed after *in ovo* PM-PSM ablation.

## 4.4 SHH, FGF AND RA PATHWAYS INTERPLAY TO REGULATE SOMITE FORMATION

Somite formation is greatly delayed in the absence of Shh signaling, but from 9h to 12h of incubation the system seems to recover as an average of one somite/90 min is observed and a difference of two somites is maintained between control and experimental sides for longer incubation periods. After 24h of incubation both control and experimental PSMs are completely segmented indicating that notochord deprivation only transiently delays somite formation. Molecular analysis of the PSM after 9h incubation indicates that this recuperation is mediated by an up-regulation of *raldh2*. Accordingly, an external RA supply to notochord deprived explants is able to rescue timely somite formation. In the absence of the notochord there is an upregulation of both *gli2* and *gli3*, which in the absence of Shh act as transcriptional repressors. RA has been shown to attenuate Gli activity (Franco et al., 1999; Goyette et al., 2000) and thus we propose that increased RA levels after 9h of notochord ablation inhibit augmented Gli2/3 activity in the PSM, rescuing somite formation. It has been shown that RA inhibits *gli2* expression (Ribes et al., 2009) and although we did not observe this effect in our experimental time frame, it is possible that somite formation recovery may involve *gli2* inhibition later in time. Taken together, these results suggest that Shh and RA pathways interplay in the PSM to regulate Gli activity and ensure timely somite formation. Interestingly, it has been described that Shh is essential for timely neural progenitor differentiation, and in this developmental system RA was also proposed to compensate for Shh deficiency (Oh et al., 2009).

Along the PSM A-P axis, a gradient of *ptch1* expression is observed: M-PSM behind the determination front presents lower expression while *ptch1* levels are higher in the determined PSM. This suggests that the determination front promotes a molecular switch in the PSM cells triggering their ability to answer to the Shh pathway. In fact, incubation of preneural tube explants with Fgf4 has been shown to inhibit the expression of ventral neural tube genes induced by Shh, suggesting that Fgf interferes with Shh or with its downstream pathway (Diez del Corral et al., 2003). It has been proposed that as Fgf levels decrease at the determination front, Shh increases and acts in concert with Wnt8c to promote expression of *raldh2*, thus activating RA (Olivera-Martinez and Storey, 2007). Accordingly, Wnt signaling in the anterior PSM induces the expression of the Shh downstream effectors *gli2* and *gli3* (Borycki et al., 2000). Taken together, these results suggest that at the determination front region, Fgf and Wnt act in concert to allow increased response to Shh signaling pathway in the PSM, thus making it competent for timely somite formation. The increased Shh activity observed above the determination front is probably regulated by Wnt and RA. It has been evidenced that proper RA levels are required for correct Shh activity. In fact, a RARE element was identified in *shh* promoter, indicating that this gene is transcriptionaly regulated by RARs (Chang et al., 1997). *Gli1* and *ptch1* expression are markedly downregulated in *radlh2*$^{-/-}$ embryos and these tissues are not able to respond to exogenous Shh supply (Ribes et al., 2006; Ribes et al., 2009). Moreover, embryo exposure to teratogenic RA doses induces severe phenotypes that include truncated limb development, accompanied by downregulation of *shh* and *ptch1* expression (Helms et al., 1997). Taken together, these results suggest that physiological RA concentrations are required to regulate Shh activity and either very low or extremely high concentrations negatively regulate the pathway. Moreover, a temporal regulation seems also to occur because Shh signaling is only downregulated after a 30h exposure to teratogenic RA doses (Helms et al., 1997). Together, this suggests that Shh and RA in the PSM act to regulate somite segmentation and molecular clock oscillations.

We observed that somite formation delay upon Shh inhibition is dose dependent, as fewer somites were formed with increased inhibitor concentrations. Moreover, we show that in the absence of Shh signaling the delay in somite formation is accompanied by an increased gene oscillation period (from 90min to 2h45min) and *raldh2* overexpression. Recently, a slower somite segmentation speed has been observed to underly the formation of the last 5-8 somites in the chick, which require 150min to form (Tenin et al., 2010). This increased time period is accompanied by a reduction and disappearance of *fgf8* and *wnt3a* expression and a surprising *raldh2* onset in the tail bud. The observed alterations in molecular gene expression and somite formation period are similar to the ones obtained in the absence of Shh. We thus suggest that the

termination of somitogenesis could involve decreased Shh activity, progressively increasing the time required for somite formation. It is tempting to postulate that during embryo development an anterior-to-posterior gradient of Shh activity could account for the different segmentation rates observed for rostral (early) and caudal (late) somites.

## 4.5 SHH SIGNALING MODULATES MOLECULAR CLOCK PERIODICITY

In the absence of the notochord, both determination front defining genes and molecular clock gene expression are affected. The embryo's caudal expression of *fgf8* is downregulated after 4.5h of incubation whereas *raldh2* upregulation is observed only after 9h of incubation. The observed delay on somite formation was not solely due to Fgf signaling downregulation because Fgf8 supplementation in notochord ablated explants did not rescue somite formation. Moreover, Fgf inhibition using SU5402 did not mimic Shh absence. Even though *fgf8* levels in the PSM are drastically reduced we never observed an increase in the somite A-P size, as was previously described to occur (Dubrulle et al., 2001). This is probably because the segmentation clock period in these conditions is severely increased, from 90min to ~2h45min. A concomitant alteration in the clock periodicity and in the determination front thus allows the formation of normal sized somites, further demonstrating that the confrontation between these two molecular mechanisms is required to regulate somite formation.

A delay in the somitogenesis molecular clock was observed both in explants cultured without notochord and when the Shh pathway was chemically inhibited. This suggests that Shh signaling acting in the PSM is regulating the pace of the molecular clock and consequently timely somite formation. Isolated L-PSM explants not only do not present somites but strikingly lose the expression of the molecular clock genes *hairy1*, *hairy2* and *lfng* (Freitas et al., 2001). Since only the M-PSM cells are directly esposed to notochord-derived Shh, an additional role of Shh in the PSM can be to ensure the molecular continuity between M- and L- compartments. In fact, a careful analysis of the anterior *hairy1* gene stripes in control PSMs pointed to the existence of a slight asynchrony in gene upregulation between M- and L- PSM (Freitas et al., 2001). This is consistent with the fact that M and L cells have distinct A-P origins in the prospective PSM territory (Selleck and Stern, 1991; Schoenwolf et al., 1992; Psychoyos and Stern, 1996; Sawada and Aoyama, 1999; Freitas et al., 2001; Eloy-Trinquet and Nicolas, 2002; Iimura et al., 2007) and further indicates that a medial-to-lateral signal is operating in the regulation of the clock expression in the PSM. The analysis of molecular clock genes in embryos in which either a

permeable or an impermeable barrier separates M- and L- compartments would indicate whether a diffusible signal is synchronizing the clock oscillations along the M-L PSM axis. Clock gene expression should also be analyzed in L-PSM explants cultured in the presence of a Shh source. We and others have previously evidenced the dynamic expression of the molecular clock genes *hairy1*, *hairy2* and *lfng* in the presumptive PSM territory (Freitas et al., 2001; Jouve et al., 2002). This showed that the PSM cells do not experience only 12 pulses of oscillatory expression prior segmentation (Palmeirim et al., 1997) and suggested that the number of cycles which a cell undergoes may determine its future A-P position. The molecular clock is thought to constitute an intrinsic counting mechanism for PSM cells, supplying endogenous information of when to integrate a somite (Palmeirim et al., 1997). The cells can probably use the same system to perceive time of exposure to Shh, transducing concentration and temporal information into distinct transcriptional activation. The molecular clock is operating along the A-P axis of both P-PSM and PSM. Note that the prospective PSM A-P axis corresponds to the future M-L axis in the PSM. This together with the observation of *hairy1* upregulation asynchrony along the M-L PSM indicates that the molecular clock provides positional information in two directions: A-P and M-L (Freitas et al., 2001). We observe graded Shh activity in both axes, thus suggesting that Shh and the clock are acting in concert to provide cells with intrinsic positional and temporal information. In fact, *hairy2* was shown to have an oscillatory behavior in the limb chondrogenic precursor cells, close to the Shh source (Pascoal et al., 2007).

Analysis of the genes regulated by Shh at the transcriptional level support our observation that Shh signaling regulates the pace of the clock. Using multipotent mesodermal cells, a series of Shh target genes were identified including *hes1* (Ingram et al., 2008). This regulation was shown to be independent of Notch signaling since DAPT treatment did not prevent *hes1* expression and RBPJk activation was not observed. Cyclopamine however prevented *hes1* activation, suggesting that it is induced by Smo downstream effectors. A similar regulation of *hes1* and *hes5* was observed in mouse retina (Wall et al., 2009). In this tissue, Shh dependent induction of *hes1* is also independent of Notch signaling whereas *hes5* activation requires functional RBPJk. This work further showed that Gli2 directly binds *hes1* promoter, indicating that it is a direct transcriptional target of the Shh pathway. Interestingly, a recent report shows that in glioblastomas Hes1 directly binds *gli1* promoter thus activating the Shh pathway (Schreck et al., 2010). This strongly suggests the existence of feedback regulatory mechanisms between these two pathways. To better understand the interaction between Notch and Shh in the PSM, a detailed analysis would be required. Due to the oscillatory behavior of the Notch genes in this tissue, it would be difficult to identify possible regulatory mechanisms *in vivo*. One alternative way to perform this study would

be to use dissociated PSM cells synchronized for clock oscillations by serum treatment (Hirata et al., 2002; Masamizu et al., 2006), interfere with Shh signaling and analyze the effects on the molecular clock oscillations by quantitative PCR (qPCR). Inhibition of Shh can be achieved either by incubation with cyclopamine or transfection with Ptch1$^{\Delta loop2}$ or GliR constructs while incubation with ShhN, SAG or GliA constructs transfection induces Shh activation (Wang et al., 2000; Briscoe et al., 2001; Chen et al., 2002; Stamataki et al., 2005; Ulloa et al., 2007). Furthermore, chromatin immunoprecipitation (chip) analysis would be required to identify putative direct Gli-mediated regulations at the promoter level of the clock genes. Since there are no commercial antibodies for chick Gli proteins, an alternative approach would be to use the well characterized C3H10T1/2 mouse fibroblasts (Hirata et al., 2002; Masamizu et al., 2006).

Iinhibition of the Wnt signaling has also been described to perturb the molecular clock period (Gibb et al., 2009). Exposing chick explants to CKI-7, an inhibitor of CK1, or to the soluble Frizzled receptor protein, which sequesters the endogenous ligand, the clock oscillation period became 115-120min rather than 90min. After 4h of incubation a few explants formed one less somite boundary than the counterlateral control (Gibb et al., 2009). We therefore analyzed the state of the Wnt signaling pathway in notochord ablated cultured explants using the downstream *axin2* gene as readout of the pathway activity. After 4.5h of incubation, *axin2* expression is unaltered in 60% of the notochord-ablated explants and the remaining 40% present only a slight downregulation of the gene. A perturbation in clock gene expression was observed in a much higher percentage of cases (80%), suggesting that somite formation delay in the absence of Shh is not primarily due to an impairment of Wnt signaling. After 9h of incubation *axin2* is clearly down-regulated in all explants. This is a predictable consequence of the absence of Shh signaling, which leads to an accumulation of GliR forms in the PSM. In fact, it has been shown that in Shh mutants *axin2* expression is reduced. In the absence of Shh signaling, high Gli3R levels inhibit the Wnt pathway (Ulloa et al., 2007; Alvarez-Medina et al., 2009), which is achieved by the direct binding of Gli3R to β-catenin (Ulloa et al., 2007). These reports indicate that Shh is capable of activating Wnt signaling and it has also been shown that Wnt can induce Gli3R forms to inhibit Shh (Alvarez-Medina et al., 2008). Overall, our results strongly suggest that Wnt signaling is not the main cause of the segmentation delay observed in the absence of Shh. In fact, our data seems to support the previous observations that Shh acts upstream of Wnt signaling.

## 4.6 HOW DOES SHH REGULATE THE CLOCK PERIODICITY?

The oscillatory behavior of the molecular clock genes relies on negative feedback loops and short lived proteins and mRNAs. Alterations on protein synthesis and degradation rates should change the clock periodicity, which in fact has been predicted by mathematical modeling (Hirata et al., 2002; Momiji and Monk, 2008). One possible explanation for the increased clock period observed in notochord ablated explants is that Shh may be affecting the protein stability of essential components of the clock machinery.

Alterations in either Shh or Wnt pathways were shown to alter the clock periodicity (our observations and Gibb et al., 2009). In the absence of either Shh or Wnt ligands, GSK3β kinase is activated phosphorylating full-length Gli or β-catenin, respectively (reviewed in (Huangfu and Anderson, 2006; Hayward et al., 2008; Varjosalo and Taipale, 2008). GSK3β has been shown to bind and phosphorylate the intracellular domain of Notch 1 and 2 receptors, with different effects on NICD stability (Foltz et al., 2002; Espinosa et al., 2003). Notch2 phosphorylation inhibits the transcriptional activation of its targets and inhibition of GSK3β activity by addition of Wnt or LiCl induces *hes1* upregulation (Espinosa et al., 2003). On the other hand, GSK3β-dependent Notch1 phosphorylation promotes Notch signaling by increasing NICD domain stability, as judged by pulse-chase experiments (Foltz et al., 2002). There are no evidences for NICD signaling regulation by GSK3β *in vivo*. Furthermore, it is not known whether the Hes/Hairy proteins are phosphorylated and what kinases might mediate this post-translational modification. It has been shown that oscillatory levels of Stat3 protein phosphorylation are required for Hes1 mRNA and protein oscillations. Both Stat3 sustained activation and inactivation abolished *hes1* oscillations, suggesting that Stat3 controls the half-life of Hes1 protein (Yoshiura et al., 2007). This was shown using serum stimulated mouse fibroblasts and it is not known if this also occurs in the PSM. Hence, alterations in protein stability due to phosphorylation levels could be one of the mechanisms controlling clock periodicity.

It would be interesting to establish whether Shh regulates the stability of the clock components NCID, Hairy2 and Lfng. Analysis of protein levels in explants with or without notochord, both by immunostaining and western blot, would indicate whether in the absence of Shh molecular clock proteins are stabilized and accumulated in the PSM. If PSM explants do not render enough protein extracts to allow an accurate evaluation, the same procedure can be use in dissociated chick PSM cells synchronized for clock oscillations (Hirata et al., 2002; Masamizu et al., 2006). If we verify a protein accumulation in the PSM following Shh inhibition, it would be important to analyze the effect of GSK3β inhibition on protein stability. Explants would be

cultured in medium supplied with either LiCl or exogenous Wnt to inhibit GSK3β activity and protein levels evaluated by western blot. This could indicate the levels of regulation exerted by Wnt and Shh pathways in Notch protein levels. If our hypothesis is correct and in the absence of Shh protein stability is increased due to increased GSK3β activity, it would be expected that increasingly high Shh levels (inhibiting GSK3β activity) induced the opposite effect. However, in our experimental conditions, we never observed an acceleration of the molecular clock, even after explant treatment with very high concentrations of Shh. This indicates that NICD stability is probably regulated by a pool of kinases that are able to compensate for GSK3β absence. In fact, NICD phosphorylation was still observed in GSK3β null cells (Foltz et al., 2002).

By regulating Notch phosphorylation levels, GSK3β can also be implicated in the intercellular coupling shown to occur in the PSM. Dissociated PSM cells gradually become out of synchrony, with the cycles' period and amplitude varying greatly between cells (Maroto et al., 2005; Masamizu et al., 2006). Both i*n vivo* and *in silico* experiments suggest that Notch signaling is responsible for intercellular communication, maintaining cell-cell synchronized oscillations in the PSM (Horikawa et al., 2006). In fact, disruption of the Dll-Notch coupling in zebrafish embryos induced lost of synchrony and increased somitogenesis period (Herrgen et al., 2010). If this would be the case, we would expect to observe a salt-and-pepper expression for the oscillatory genes in the absence of Shh, indicative that cells still cycle individually but lost their overall synchrony. This is not the case for *hairy2* and *lfng* expressions and thus we think that the regulatory Shh function is mediated by a different mechanism.

## 4.7 A MODEL FOR SHH ACTIVITY IN VERTEBRATE EMBRYO SOMITOGENESIS

Taken together, the results presented throughout this thesis suggest that Shh signaling is required for proper embryo somitogenesis due to its regulatory activities in distinct events including PSM specification, molecular clock periodicity and timely somite formation as well as recruitment of lateral cells for incorporation in each somite. We thus propose the following explanatory model for the different Shh activities in this embryonic mechanism (see Fig. 4.1). In the prospective PSM territory an A-P gradient of Shh activity is established, probably through Ptch2, with the PM being under the influence of high Shh levels while PL receives less ligand. Thus, a dose dependent mechanism seems to specify medial and lateral PSM cell fates. Molecular clock oscillations have been shown to occur in this region (Freitas et al., 2001; Jouve et al., 2002) and Shh might be regulating its periodicity. As the embryo grows and prospective cells leave the

tailbud region and progressively occupy more anterior locations, M- and L- cells are continuously exposed to distinct levels of Shh signaling: M cells are adjacent to the midline structures and express low *ptch1* levels while L ones probably do not perceive any Shh. It has been shown that in the undetermined PSM, Shh signalling is inhibited by high Fgf levels (Diez del Corral et al., 2003; Ribes et al., 2009) but some residual activity of the pathway must still occur since our results indicate that in Shh absence the segmentation molecular clock period is increased in this region. As Fgf levels progressively decrease nearing the determination front, its inhibitory effect on Shh is relieved and Shh together with Wnt8c (induced by Fgf) promote *raldh2* expression. Above the determination front, RA and Wnt are probably involved in activating the Shh pathway by regulating the expression of its downstream effectors (Helms et al., 1997; Borycki et al., 2000; Ribes et al., 2006; Ribes et al., 2009). In the determined PSM, a gradient of *ptch1* expression is observed with higher levels in its medial-anterior most region. This probably establishes a gradient of Shh activity along both the A-P and M-L axis of the determined PSM. Somite formation has been shown to start from the medial-most PSM cells (Martins et al., 2009) and we propose that this is mediated by Shh. M-PSM cells have high *ptch1* expression, indicative of an active response to Shh and seem to be responsible for recruiting more lateraly located cells to integrate a somite. Due to cell autonomous and non-autonomous feedback mechanisms (reviewed in Dessaud et al., 2008; Ribes and Briscoe, 2009), Shh signal is propagated along the M-L axis inducing the complex cell rearrangements required for somite formation (Kulesa and Fraser, 2002; Martins et al., 2009). The M-L graded activity of Shh is presumably counteracted by an opposing signal from the lateral plate mesoderm. Our observations indicate that the medial PSM cells are under the influence of higher Shh concentrations and for a longer period of time than the lateral domain, which probably is essential for their specification. As the embryo elongates caudally, new cells are allocated to the anterior most PSM and the same molecular and morphologic events take place to form a new somite.

## 4.8 CONCLUDING REMARKS

The Hh signaling family has been implicated in several mechanisms during embryo development. In vertebrates, Shh is the most studied Hh member and it has been shown to play key functions in the patterning and specification of cells, such as in the limb bud and in the ventral neural tube (reviewed in Varjosalo and Taipale, 2008). The severe phenotypes presented by Shh knock-out mice are indicative of the importance of this molecule for proper development and include growth retardation and lack of distinct fore- and hind- limbs. Moreover, mutants lack the

entire vertebrate column and present only five to six ribs (Chiang et al., 1996). Shh implication in somite sclerotome induction (Fan and Tessier-Lavignet, 1994) and in survival of medial somitic cells (Rong et al., 1992; Teillet et al., 1998) has been considered to account for the KO phenotype. In the present work we addressed the midline Shh derived contribution to vertebrate embryo somitogenesis and our results suggest that an additional perturbation in somite segmentation could be contributing to the mutant's phenotype. We present evidence supporting a role for Shh pathway in PSM specification, regulation of timely somite formation and maintenance of periodic molecular clock oscillations, implicating this morphogen in the somitogenesis machinery for the first time.

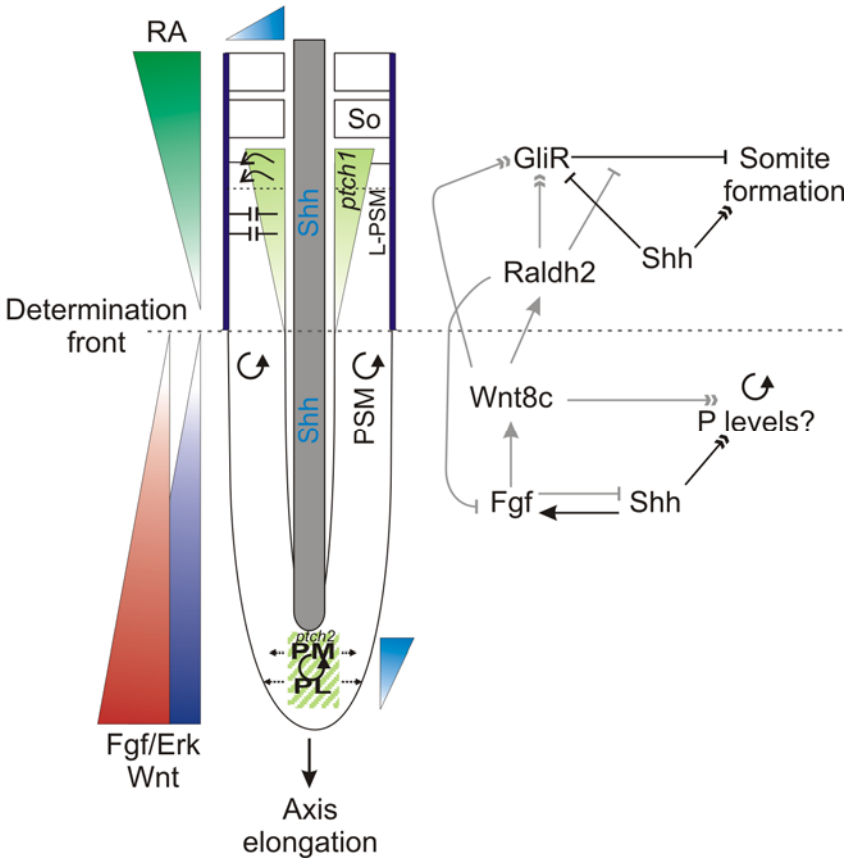



**Figure 4.1. Model for Shh activity in embryo PSM.** Schematic representation of the Shh signaling activities in the presomitic mesoderm (PSM). Midline derived Shh is perceived by P-PSM cells (green striped square) probably through Ptch2 receptor establishing a gradient of Shh activity (blue triangle) along the A-P axis of the presumptive PSM territory. This graded Shh activity is presumably responsible for M- and L specification and for regulation of the molecular clock oscillations (black spiral symbol) observed in this region. As the embryo grows, P-PSM cells progressively occupy more anterior regions and medial cells, being juxtaposed to the midline structures are probably constinuosly under the influence of residual Shh activity. High Fgf levels present in the undetermined PSM (red

triangle) inhibit Shh (Diez del Corral et al., 2003; Ribes et al., 2009) and induce Wnt8c (purple triangle) (Olivera-Martinez and Storey, 2007). Shh and Wnt possibily act together to regulate molecular clock oscillations periodicity by regulating protein phosphorylation levels (P levels). As Fgf levels drop when approaching the determination front, Shh levels increase and together with Wnt8c induce *raldh2* expression and thus RA activity (RA, green triangle) (our observations and Olivera-Martinez and Storey, 2007). In the determined PSM region, Gli activity is regulated by both Wnt and RA signaling (Borycki et al., 2000; Ribes et al., 2009), which allows increased Shh activity. In the anterior PSM, Shh inhibits GliR activity and regulates morphological somite formation: medial PSM cells expressing *ptch1* are actively responding to Shh from the notochord. A gradient of Shh activity is established along the M-L axis of the anterior PSM, which is probably required for the recruitment of lateral cells by medial ones to integrate a somite (dashed line). Shh activity in this region is probably counteracted by an unknown signal from the lateral plate mesoderm (purple). PM and PL represents prospective medial and lateral PSM, respectively; arrows indicate future cell position in the PSM. SI, last formed somite. Grey lines indicate interactions shown elsewhere and black lines those established in the present work. Solid arrowheads indicate transcriptional activation; double-head arrows represent a regulatory action.

Somite formation is characteristic of all vertebrate species and the segmented structures arising from them are essential for the high flexibility and mobility of the adult body. Somitogenesis is underlined by a complex regulatory network that includes members of distinct signaling pathways such as Fgf, Wnt, RA and Notch. The molecular machinery regulating somite formation is conserved among species, including zebrafish, chick, mouse, frog and snakes (Andrade et al., 2007; Gomez et al., 2008). Several studies have tried to understand how exactly the distinct pathways operate to control somite formation and even though some interactions have been identified it is still not clear how these signaling are integrated. With the present work we implicate Shh in this intricate molecular machinery regulating correct somite formation. In fact, in both limb and neural development, Shh has been shown to interact with Fgf, Wnt and RA to regulate cellular responses and patterning. By identifying a similar interplay operating during somitogenesis, we propose that these interactions are conserved between distinct developmental systems. How exactly this is achieved is not yet clear. Congenital vertebral malformations have been associated with alterations in either Notch (reviewed in Andrade et al., 2007; Turnpenny et al., 2007; Giampietro et al., 2009) and Shh (Kim et al., 2001; Celli et al., 2003) pathways. Elucidation of the molecular mechanisms regulating pathway mediators in thus required.

## 4.9 FUTURE PERSPECTIVES

The working model presented in Fig. 4.1 contains several interesting questions and proposals that are tempting to further investigate.

We show that the prospective PSM domains are differently committed for somite formation and suggest that this is mediated by Shh signaling. Implantation of a bead soaked in cyclopamine in the PM region and analyses of the number of somites formed after incubation would clearly show whether Shh is indeed required for M specification. Alternatively, chick/quail grafts experiments could be performed to generate chimeric embryos with two PL domains. A cyclopamine-soaked bead placed on the quail PL (now in the PM position) would indicate whether Shh is the signal responsible for their re-specification. Moreover, it would be interesting to analyze the state of the molecular clock oscillations in the presumptive PSM after Shh inhibition. Distribution of the Shh protein along the P-PSM A-P axis in control and inhibited conditions should also be assessed.

Isolated L-PSM explants not only do not form somites but also lose the expression of molecular clock genes (Freitas et al., 2001), suggesting that a gradient of Shh regulates gene expression along the M-L axis. Insertion of a permeable or impermeable barrier separating M- and L- PSM compartments *in ovo* followed by molecular clock gene expression analysis would demonstrate if a diffusible molecule regulates M-L clock expression. Incubation of L-PSM explants with a Shh source would further show whether that signal is Shh. As in the P-PSM, Shh protein expression should be evaluated in the determined PSM in both control and L-PSM isolated explants. Moreover, embryo electroporation with the Ptch1$^{\Delta loop2}$ construct (Briscoe et al., 2001) would indicate if the medial PSM is indeed specified by a continuous Shh signaling and if anterior morphologic somite formation relies on the graded *ptch1* expression. *In situ* hybridization analysis would also be required to identify the putative signal from the lateral plate opposing Shh activity in the anterior PSM.

We observe a delay in the molecular clock oscillatory period after Shh deprivation and hypothesise that the clock periodicity is regulated by different phosphorylation levels that alter the stability of the oscillatory proteins. To demonstrate if this is the case, protein levels in explants with and without notochord or in synchronized PSM cells treated with either DMSO or cyclopamine should be analyzed. We expect to observe a protein accumulation in the absence of Shh, indicative of higher protein stability. Performing the same kind of analysis after GSK3β inhibition using either LiCl or Wnt protein, will show whether the Shh induced increased periodicity is mediated by GSK3β. If this is the case, it is indicative of a synergistic effect of Wnt and Shh to regulate Notch oscillations. In multipotent mesodermal cells (Ingram et al., 2008) and mouse retina (Wall et al., 2009), Shh has been shown to regulate *hes1* expression in a Notch independent manner. This is thought to be mediated by direct Gli2 binding to *hes1* promoter (Wall

et al., 2009). To evaluate if the same regulation is occurring in the PSM synchronized PSM dissociated cells could be use. Shh signaling in these cells could be inhibited or activated using previously described constructs (Wang et al., 2000; Briscoe et al., 2001; Stamataki et al., 2005; Ulloa et al., 2007) and the effect on the Notch genes *hairy1*, *hairy2* and *lfng* oscillatory expression assessed by qPCR. Chromatin immunoprecipitation assays would identify putative regulations between Shh and Notch pathways at the promoter level. This analysis is made harder to carry out in chick since there are no commercial antibodies available for chick Gli proteins. For this reason, this approach could be performed in C3H10T1/2 mouse fibroblasts (Hirata et al., 2002; Masamizu et al., 2006).

The suggested experiments would provide further evidence for a Shh role in both somite formation and gene oscillation regulation. They would furthermore help clarify at what level of the signal transduction machinery Notch and Shh pathways crosstalk. We thus hope that these studies contribute to a better comprehension of the intricate molecular regulations occurring during somitogenesis.

## 4.10 REFERENCES

Alvarez-Medina, R., Cayuso, J., Okubo, T., Takada, S. and Marti, E. (2008) 'Wnt canonical pathway restricts graded Shh/Gli patterning activity through the regulation of Gli3 expression', *Development* 135(2): 237-47.

Alvarez-Medina, R., Le Dreau, G., Ros, M. and Marti, E. (2009) 'Hedgehog activation is required upstream of Wnt signalling to control neural progenitor proliferation', *Development* 136(19): 3301-9.

Andrade, R. P., Palmeirim, I. and Bajanca, F. (2007) 'Molecular clocks underlying vertebrate embryo segmentation: A 10-year-old hairy-go-round', *Birth Defects Res C Embryo Today* 81(2): 65-83.

Borycki, A.-G., Brown, A. M. C. and Emerson, J. C. P. (2000) 'Shh and Wnt signaling pathways converge to control Gli gene activation in avian somite', *Development* 127: 2075-2087.

Briscoe, J., Chen, Y., Jessell, T. M. and Struhl, G. (2001) 'A Hedgehog-Insensitive Form of Patched Provides Evidence for Direct Long-Range Morphogen Activity of Sonic Hedgehog in the Neural Tube', *Molecular Cell* 7: 1279-1291.

Cairns, D. M., Sato, M. E., Lee, P. G., Lassar, A. B. and Zeng, L. (2008) 'A gradient of Shh establishes mutually repressing somitic cell fates induced by Nkx3.2 and Pax3', *Dev Biol* 323(2): 152-65.

Catala, M., Teillet, M. A., De Robertis, E. M. and Le Douarin, M. L. (1996) 'A spinal cord fate map in the avian embryo: while regressing, Hensen's node lays down the notochord and floor plate thus joining the spinal cord lateral walls', *Development* 122(9): 2599-610.

Catala, M., Teillet, M. A. and Le Douarin, N. M. (1995) 'Organization and development of the tail bud analyzed with the quail-chick chimaera system', *Mech Dev* 51(1): 51-65.

Celli, J., van Bokhoven, H. and Brunner, H. G. (2003) 'Feingold syndrome: clinical review and genetic mapping', *Am J Med Genet A* 122A(4): 294-300.

Chang, B. E., Blader, P., Fischer, N., Ingham, P. W. and Strahle, U. (1997) 'Axial (HNF3beta) and retinoic acid receptors are regulators of the zebrafish sonic hedgehog promoter', *EMBO J* 16(13): 3955-64.

Chen, J. K., Taipale, J., Young, K. E., Maiti, T. and Beachy, P. A. (2002) 'Small molecule modulation of Smoothened activity', *Proc Natl Acad Sci U S A* 99(22): 14071-6.

Chen, Y. and Struhl, G. (1996) 'Dual Roles for Patched in Sequestering and Transducing Hedgehog', *Cell* 87: 553-563.

Chiang, C., Litingtung, Y., Lee, E., Young, K. E., Corden, J. L., Westphal, H. and Beachy, P. A. (1996) 'Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function', *Nature* 383(6599): 407-13.

Dessaud, E., McMahon, A. P. and Briscoe, J. (2008) 'Pattern formation in the vertebrate neural tube: a sonic hedgehog morphogen-regulated transcriptional network', *Development* 135(15): 2489-503.

Dessaud, E., Ribes, V., Balaskas, N., Yang, L. L., Pierani, A., Kicheva, A., Novitch, B. G., Briscoe, J. and Sasai, N. (2010) 'Dynamic assignment and maintenance of positional identity in the ventral neural tube by the morphogen sonic hedgehog', *PLoS Biol* 8(6): e1000382.

Dessaud, E., Yang, L. L., Hill, K., Cox, B., Ulloa, F., Ribeiro, A., Mynett, A., Novitch, B. G. and Briscoe, J. (2007) 'Interpretation of the sonic hedgehog morphogen gradient by a temporal adaptation mechanism', *Nature* 450(7170): 717-20.

Diez del Corral, R., Olivera-Martinez, I., Goriely, A., Gale, E., Maden, M. and Storey, K. (2003) 'Opposing FGF and retinoid pathways control ventral neural pattern, neuronal differentiation, and segmentation during body axis extension', *Neuron* 40(1): 65-79.

Dubrulle, J., McGrew, M. J. and Pourquie, O. (2001) 'FGF signaling controls somite boundary position and regulates segmentation clock control of spatiotemporal Hox gene activation', *Cell* 106(2): 219-232.

Eloy-Trinquet, S. and Nicolas, J. F. (2002) 'Cell coherence during production of the presomitic mesoderm and somitogenesis in the mouse embryo', *Development* 129(15): 3609-3619.

Espinosa, L., Ingles-Esteve, J., Aguilera, C. and Bigas, A. (2003) 'Phosphorylation by glycogen synthase kinase-3 beta down-regulates Notch activity, a link for Notch and Wnt pathways', *J Biol Chem* 278(34): 32227-35.

Fan, C.-M. and Tessier-Lavignet, M. (1994) 'Patterning of mammalian somites by surface ectoderm and notochord: evidence for sclerotome induction by a hedgehog homolog', *Cell* 79(7): 1175-8.

Foltz, D. R., Santiago, M. C., Berechid, B. E. and Nye, J. S. (2002) 'Glycogen synthase kinase-3beta modulates notch signaling and stability', *Curr Biol* 12(12): 1006-11.

Franco, P. G., Paganelli, A. R., Lopez, S. L. and Carrasco, A. E. (1999) 'Functional association of retinoic acid and hedgehog signaling in Xenopus primary neurogenesis', *Development* 126(19): 4257-65.

Freitas, C., Rodrigues, S., Charrier, J. B., Teillet, M. A. and Palmeirim, I. (2001) 'Evidence for medial/lateral specification and positional information within the presomitic mesoderm', *Development* 128(24): 5139-47.

Giampietro, P. F., Dunwoodie, S. L., Kusumi, K., Pourquie, O., Tassy, O., Offiah, A. C., Cornier, A. S., Alman, B. A., Blank, R. D., Raggio, C. L. et al. (2009) 'Progress in the understanding of the genetic etiology of vertebral segmentation disorders in humans', *Ann N Y Acad Sci* 1151: 38-67.

Gibb, S., Zagorska, A., Melton, K., Tenin, G., Vacca, I., Trainor, P., Maroto, M. and Dale, K. (2009) 'Interfering with Wnt signalling alters the periodicity of the segmentation clock', *Developmental Biology* 330(1): 21-31.

Gomez, C., Ozbudak, E. M., Wunderlich, J., Baumann, D., Lewis, J. and Pourquie, O. (2008) 'Control of segment number in vertebrate embryos', *Nature* 454(7202): 335-9.

Goyette, P., Allan, D., Peschard, P., Chen, C. F., Wang, W. and Lohnes, D. (2000) 'Regulation of gli activity by all-trans retinoic acid in mouse keratinocytes', *Cancer research* 60(19): 5386-5389.

Hayward, P., Kalmar, T. and Arias, A. M. (2008) 'Wnt/Notch signalling and information processing during development', *Development* 135(3): 411-24.

Helms, J. A., Kim, C. H., Hu, D., Minkoff, R., Thaller, C. and Eichele, G. (1997) 'Sonic hedgehog participates in craniofacial morphogenesis and is down-regulated by teratogenic doses of retinoic acid', *Dev Biol* 187(1): 25-35.

Herrgen, L., Ares, S., Morelli, L. G., Schroter, C., Julicher, F. and Oates, A. C. (2010) 'Intercellular coupling regulates the period of the segmentation clock', *Curr Biol* 20(14): 1244-53.

Hirata, H., Yoshiura, S., Ohtsuka, T., Bessho, Y., Harada, T., Yoshikawa, K. and Kageyama, R. (2002) 'Oscillatory expression of the bHLH factor Hes1 regulated by a negative feedback loop', *SCIENCE* 298(5594): 840-843.

Horikawa, K., Ishimatsu, K., Yoshimoto, E., Kondo, S. and Takeda, H. (2006) 'Noise-resistant and synchronized oscillation of the segmentation clock', *Nature* 441(7094): 719-23.

Huangfu, D. and Anderson, K. V. (2006) 'Signaling from Smo to Ci/Gli: conservation and divergence of Hedgehog pathways from Drosophila to vertebrates', *Development* 133(1): 3-14.

Iimura, T., Yang, X., Weijer, C. J. and Pourquie, O. (2007) 'Dual mode of paraxial mesoderm formation during chick gastrulation', *Proc Natl Acad Sci U S A* 104(8): 2744-9.

Incardona, J. P., Gaffield, W., Kapur, R. P. and Roelink, H. (1998) 'The teratogenic Veratrum alkaloid cyclopamine inhibits sonic hedgehog signal transduction', *Development* 125(18): 3553-62.

Ingram, W. J., McCue, K. I., Tran, T. H., Hallahan, A. R. and Wainwright, B. J. (2008) 'Sonic Hedgehog regulates Hes1 through a novel mechanism that is independent of canonical Notch pathway signalling', *Oncogene* 27(10): 1489-500.

Jarov, A., Williams, K. P., Ling, L. E., Koteliansky, V. E., Duband, J. L. and Fournier-Thibault, C. (2003) 'A dual role for Sonic hedgehog in regulating adhesion and differentiation of neuroepithelial cells', *Dev.Biol.* 261(2): 520-536.

Jeong, J. and McMahon, A. P. (2005) 'Growth and pattern of the mammalian neural tube are governed by partially overlapping feedback activities of the hedgehog antagonists patched 1 and Hhip1', *Development* 132(1): 143-54.

Jouve, C., Iimura, T. and Pourquie, O. (2002) 'Onset of the segmentation clock in the chick embryo: evidence for oscillations in the somite precursors in the primitive streak', *Development* 129(5): 1107-1117.

Kawakami, T., Kawcak, T., Li, Y. J., Zhang, W., Hu, Y. and Chuang, P. T. (2002) 'Mouse dispatched mutants fail to distribute hedgehog proteins and are defective in hedgehog signaling', *Development* 129(24): 5753-65.

Kim, P. C., Mo, R. and Hui Cc, C. (2001) 'Murine models of VACTERL syndrome: Role of sonic hedgehog signaling pathway', *J Pediatr Surg* 36(2): 381-4.

Kulesa, P. M. and Fraser, S. E. (2002) 'Cell dynamics during somite boundary formation revealed by time-lapse analysis', *SCIENCE* 298(5595): 991-995.

Maroto, M., Dale, J. K., Dequeant, M. L., Petit, A. C. and Pourquie, O. (2005) 'Synchronised cycling gene oscillations in presomitic mesoderm cells require cell-cell contact', *Int J Dev Biol* 49(2-3): 309-15.

Martins, G. G., Rifes, P., Amandio, R., Rodrigues, G., Palmeirim, I. and Thorsteinsdottir, S. (2009) 'Dynamic 3D cell rearrangements guided by a fibronectin matrix underlie somitogenesis', *PLoS One* 4(10): e7429.

Masamizu, Y., Ohtsuka, T., Takashima, Y., Nagahara, H., Takenaka, Y., Yoshikawa, K., Okamura, H. and Kageyama, R. (2006) 'Real-time imaging of the somite segmentation clock: Revelation of unstable oscillators in the individual presomitic mesoderm cells', *Proc.Natl.Acad.Sci.U.S.A* 103(5): 1313-1318.

Momiji, H. and Monk, N. A. (2008) 'Dissecting the dynamics of the Hes1 genetic oscillator', *J Theor Biol* 254(4): 784-98.

Oh, S., Huang, X., Liu, J., Litingtung, Y. and Chiang, C. (2009) 'Shh and Gli3 activities are required for timely generation of motor neuron progenitors', *dev. Biol.* 331(2): 261-269.

Olivera-Martinez, I. and Storey, K. G. (2007) 'Wnt signals provide a timing mechanism for the FGF-retinoid differentiation switch during vertebrate body axis extension', *Development* 134(11): 2125-35.

Packard, D. S. (1980) 'Somitogenesis in Cultured Embryos of the Japanese Quail, Coturnix coturnix japonica', *The American Journal of Anatomy* 158: 83-91.

Packard, D. S. and Jacobson, A. G. (1976) 'The Influence of Axial Structures on Chick Somite Formation', *Dev.Biol.* 3: 36-48.

Packard, D. S., Jr. and Meier, S. (1983) 'An experimental study of the somitomeric organization of the avian segmental plate', *Dev Biol* 97(1): 191-202.

Packard, D. S., Jr., Zheng, R.-Z. and Turner, D. C. (1993) 'Somite pattern regulation in the avian segmental plate mesoderm', *Development* 117: 779-791.

Palmeirim, I., Henrique, D., Ish-Horowicz, D. and Pourquie, O. (1997) 'Avian hairy gene expression identifies a molecular clock linked to vertebrate segmentation and somitogenesis', *Cell* 91(5): 639-648.

Pascoal, S., Carvalho, C. R., Rodriguez-Leon, J., Delfini, M. C., Duprez, D., Thorsteinsdottir, S. and Palmeirim, I. (2007) 'A Molecular Clock Operates During Chick Autopod Proximal-distal Outgrowth', *J Mol Biol*.

Pourquie, O., Fan, C. M., Coltey, M., Hirsinger, E., Watanabe, Y., Breant, C., Francis-West, P., Brickell, P., Tessier-Lavigne, M. and Le Douarin, N. M. (1996) 'Lateral and axial signals involved in avian somite patterning: a role for BMP4', *Cell* 84(3): 461-71.

Psychoyos, D. and Stern, C. D. (1996) 'Fates and migratory routes of primitive streak cells in the chick embryo', *Development* 122(5): 1523-34.

Reshef, R., Maroto, M. and Lassar, A. B. (1998) 'Regulation of dorsal somitic cell fates: BMPs and Noggin control the timing and pattern of myogenic regulator expression', *Genes Dev* 12(3): 290-303.

Ribes, V. and Briscoe, J. (2009) 'Establishing and interpreting graded Sonic Hedgehog signaling during vertebrate neural tube patterning: the role of negative feedback', *Cold Spring Harb Perspect Biol* 1(2): a002014.

Ribes, V., Le Roux, I., Rhinn, M., Schuhbaur, B. and Dolle, P. (2009) 'Early mouse caudal development relies on crosstalk between retinoic acid, Shh and Fgf signalling pathways', *Development* 136(4): 665-76.

Ribes, V., Wang, Z., Dolle, P. and Niederreither, K. (2006) 'Retinaldehyde dehydrogenase 2 (RALDH2)-mediated retinoic acid synthesis regulates early mouse embryonic forebrain development by controlling FGF and sonic hedgehog signaling', *Development* 133(2): 351-61.

Rong, P. M., Teillet, M.-A., Ziller, C. and Le Douarin, N. M. (1992) 'The neural tube/notochord complex is necessary for vertebral but not limb and body wall striated muscle differentiation', *Development* 115: 657-672.

Sawada, K. and Aoyama, H. (1999) 'Fate maps of the primitive streak in chick and quail embryo: ingression timing of progenitor cells of each rostro-caudal axial level of somites', *Int.J Dev.Biol.* 43(8): 809-815.

Schoenwolf, G. C., Garcia-Martinez, V. and Dias, M. S. (1992) 'Mesoderm movement and fate during avian gastrulation and neurulation', *Dev Dyn* 193(3): 235-48.

Schreck, K. C., Taylor, P., Marchionni, L., Gopalakrishnan, V., Bar, E. E., Gaiano, N. and Eberhart, C. G. (2010) 'The Notch target Hes1 directly modulates Gli1 expression and Hedgehog signaling: a potential mechanism of therapeutic resistance', *Clin Cancer Res* 16(24): 6060-70.

Selleck, M. A. and Stern, C. D. (1991) 'Fate mapping and cell lineage analysis of Hensen's node in the chick embryo', *Development* 112(2): 615-26.

Stamataki, D., Ulloa, F., Tsoni, S. V., Mynett, A. and Briscoe, J. (2005) 'A gradient of Gli activity mediates graded Sonic Hedgehog signaling in the neural tube', *Genes Dev* 19(5): 626-41.

Stockdale, F. E., Nikovits, W., Jr. and Christ, B. (2000) 'Molecular and cellular biology of avian somite development', *Dev Dyn* 219(3): 304-21.

Teillet, M.-A., LAPOINTE, F. and Le Douarin, N. M. (1998) 'The relationships between notochord and floor plate in vertebrate development revisited', *Proc. Natl. Acad. Sci. USA* 95 11733-11738.

Tenin, G., Wright, D., Ferjentsik, Z., Bone, R., McGrew, M. J. and Maroto, M. (2010) 'The chick somitogenesis oscillator is arrested before all paraxial mesoderm is segmented into somites', *BMC Dev Biol* 10: 24.

Testaz, S., Jarov, A., Williams, K. P., Ling, L. E., Koteliansky, V. E., Fournier-Thibault, C. and Duband, J. L. (2001) 'Sonic hedgehog restricts adhesion and migration of neural crest cells independently of the Patched- Smoothened-Gli signaling pathway', *Proc.Natl.Acad.Sci.U.S.A* 98(22): 12521-12526.

Turnpenny, P. D., Alman, B., Cornier, A. S., Giampietro, P. F., Offiah, A., Tassy, O., Pourquie, O., Kusumi, K. and Dunwoodie, S. (2007) 'Abnormal vertebral segmentation and the notch signaling pathway in man', *Dev Dyn* 236(6): 1456-74.

Ulloa, F., Itasaki, N. and Briscoe, J. (2007) 'Inhibitory Gli3 activity negatively regulates Wnt/beta-catenin signaling', *Curr Biol* 17(6): 545-50.

Varjosalo, M. and Taipale, J. (2008) 'Hedgehog: functions and mechanisms', *Genes Dev* 22(18): 2454-72.

Wall, D. S., Mears, A. J., McNeill, B., Mazerolle, C., Thurig, S., Wang, Y., Kageyama, R. and Wallace, V. A. (2009) 'Progenitor cell proliferation in the retina is dependent on Notch-independent Sonic hedgehog/Hes1 activity', *J Cell Biol* 184(1): 101-12.

Wang, B., Fallon, J. F. and Beachy, P. A. (2000) 'Hedgehog-regulated processing of Gli3 produces an anterior/posterior repressor gradient in the developing vertebrate limb', *Cell* 100(4): 423-34.

Yoshiura, S., Ohtsuka, T., Takenaka, Y., Nagahara, H., Yoshikawa, K. and Kageyama, R. (2007) 'Ultradian oscillations of Stat, Smad, and Hes1 expression in response to serum', *Proc Natl Acad Sci U S A* 104(27): 11292-7.